Isolation of Australian Microalgae and Preparation of Microalgal Concentrates for use as Aquaculture Feeds

by

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and that, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

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Richard Knuckey

Abstract

In this study, two types of microalgal feed were examined; 1) algal concentrates and 2) new Australian microalgae. Algal concentrates were examined because for aquaculturists, they could provide an off-the-shelf alternative to maintaining live algal cultures. Australian microalgae were examined to identify new feed species and to determine specific dietary requirements of local juvenile Pacific oysters (*Crassostrea gigas*).

Ten diatoms, microalgae widely cultivated as feed for aquaculture species, were isolated and purified from local waters, Tasmania, Australia. Proximate analysis of logarithmic and stationary phase cultures showed major differences between species and in the effect of stationary phase on composition. Four species (*Attheya septentrionalis*, *Entomoneis* cf. *punctulata*, *Extubocellulus spinifera* and *Thalassiosira oceanica*) were evaluated as feed for juvenile Pacific oysters. Fed as the major component in a ternary algal diet, two algae (*Attheya septentrionalis* and *Entomoneis* cf. *punctulata*) supported over 80% of the growth of the *T. pseudonana* control.

Centrifugation of eleven microalgae from a range of classes showed it to be an efficient (>80 % recovery) way to concentrate microalgae. Small chlorophytes survived the high shear forces best but, are documented poor algal diets for oysters. Of the diatoms *Chaetoceros calcitrans* and *Thalassiosira pseudonana* were least damaged. Juvenile oyster feeding trials showed that *T. pseudonana* pastes were capable of sustaining limited growth. However, nutritional deficiencies in the pastes were reflected in falling weekly growth rates, by the third week, oysters grew only marginally or lost organic weight.

An alternative, low shear concentration process was developed based on chemical induced flocculation. Initial coagulation of algae was induced using Fe^{+3} or by increasing pH. Flocculated concentrates of *T. pseudonana* were fed to juvenile Pacific oysters and compared to live *T. pseudonana* and centrifuged algal pastes. The pH

flocculated algal diet was superior to all other test diets and lost nutritional value more slowly than centrifuged algal diets.

In the discussion the nutritional composition of algal diets is related to the composition and nutritional requirements of the juvenile oysters. It is argued that the dietary protein content of live algal diets, not limited in essential nutrients, is the most significant factor in determining a nutritionally superior diet. The nutritional value of algal concentrates is discussed and flocculation is concluded to be a superior method to produce concentrates. It is shown that flocculation is applicable for concentrating many algal species and argued that it is a cost-effective process with commercial applications.

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Table of Contents

Abbreviations

AA	ascorbic acid
AFDW	ash free dry weight
%AGE	apparent growth efficiency
ANOVA	analysis of variance
C/N	Carbon : Nitrogen ratio
CRC	Cooperative Research Centre
CRI	component retention index
CSIRO	Commonwealth Scientific Industrial Research Organisation
CV	coefficient of variation
DHA	docosahexaenoic acid 22:6 n-3
DIC	differential interference contrast
DW	dry weight
EAAI	essential amino acid index
EDTA	ethylene diamine tetra acetic acid
EPA	eicosapentaenoic acid 22:5 n-3
f/2	modified F media (Guillard and Ryther, 1962), half strength
GLM	general linear model
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HUFA	highly unsaturated fatty acid
k	instantaneous growth rate
LM	light microscope
MAV	marine agar 2216 with vitamins
MPA	3% metaphosphoric acid + 8% acetic acid
NaOH	sodium hydroxide
OPD	o-phenylenediamine
PLSD	protected least significant difference
PUFA	polyunsaturated fatty acid
S.D.	standard deviation
S.E.	standard error
SEM	scanning electron microscope
SWAV	seawater agar with vitamins
TCBS	thiosulphate-citrate-bile salt-sucrose Cholera medium
TEM	transmission electron microscope

Chapter One

Literature Review

Chapter 1

Literature Review

1.1 Microalgae in Mariculture

Microalgae are classifications of photosynthetic, aquatic plants based on size (1 μm-2 mm) with members being unicellular, colonial or filamentous. Microalgae smaller than 20 μm are further classified as nano- and pico-plankton. All algal phyta contribute members to the microalgae group. However, Rhodophyta and Phaeophyta have only a few microscopic members within their classifications. Many microalgae are motile or have aids to buoyancy and collectively they make up the phytoplankton, forming the basis of almost all marine food chains. Microalgae are widely used as feed for larval and juvenile stages of fish, crustaceans and molluscs. However, they are of greater importance to molluscs which feed almost exclusively on marine microalgae throughout their life cycle (Benemann, 1992). Coutteau and Sorgeloos (1992), conducted a survey of bivalve facilities and ranked the most commonly used microalgae, dependent on their frequency and volume of culture (Table 1.1).

Table 1.1 The ten most commonly used algae by oyster facilities (Coutteau and Sorgeloos, 1992), compared to the species that gave the best growth response for the oyster *Ostrea edulis* (Enright *et al.*, 1986a).

Algal Species Assigned Ranking		
Based on frequency of use in bivalve hatcheries. (Coutteau and Sorgeloos, 1992).		Based on growth response obtained with juvenile *Ostrea edulis* (Enright *et al.*, 1986a).
Isochrysis sp. (T-iso)	1	*Chaetoceros gracilis*
Chaetoceros gracilis	2	*Chaetoceros calcitrans*
Chaetoceros calcitrans	3	*Skeletonema costatum*
Tetraselmis suecica	4	*Chaetoceros simplex*
Thalassiosira pseudonana	5	*Rhodomonas* sp.
Pavlova lutheri	6	*Thalassiosira pseudonana*
Isochrysis galbana	7	*Isochrysis* sp. (T-iso)
Skeletonema costatum	8	*Tetraselmis maculata*
Chroomonas salina	9	*Tetraselmis* sp.
Dunaliella tertiolecta	10	*Pavlova lutheri*

Although their algal ranking generally corresponded to those that have a good nutritional value to oysters, ease of culture and tradition also influence the choice of algae cultured in hatcheries. There is variation in the literature regarding classification but ten divisions (including 2 procaryote divisions) are frequently cited. The microalgae that are most often used in mollusc culture come mainly from the Chlorophyta and Chrysophyta divisions (Table 1.2).

Table 1.2 Classification of commonly cultured eucaryote microalgae used in aquaculture.

Division	Class	Order	Family	Genus
Chlorophyta	Chlorophyceae	Volvocales	Polyblepharidaceae	Dunaliella
			Chlamydomonadaceae	Chlamydomonas Branchiomonas Micromonas Haematococcus
		Chlorococcales	Oocystaceae	Chlorella Nannochloris
	Prasinophyceae			Pyramimonas Tetraselmis *
Chrysophyta	Chrysophyceae Prymnesiophyceae	Isochrysidales		Isochrysis * Dicrateria
		Pavlovales		Pavlova *
	Eustigmatophyceae			Nannochloropsis
	Bacillariophyceae	Centrales	Thalassiosiracea	Skeletonema * Thalassiosira *
			Chaetoceraceae	Chaetoceros *
		Pennales	Cymbellaceae Bacillariaceae Naviculaceae	Phaeodactylum Nitzschia * Navicula *
Cryptophyta	Cryptophyceae			Rhodomonas * Cryptomonas Chroomonas *

* Species commonly used in mollusc culture

1.2. Biochemical Composition of Microalgae and their Nutritional Value to Molluscs

1.2 i. Gross composition of microalgae

Microalgae, like any living organism, are composed of organic molecules, broadly classified as protein, carbohydrate and lipids with an inorganic mineral fraction. The distribution between these groups is dependent on the general conditions of culture and the status of the algae. Factors such as light intensity and photoperiod, growth stage, temperature, pH and particularly nutrient status all effect the final gross composition (Fabregas *et al.*, 1986; Millamena *et al.*, 1990b; Renaud *et al.*, 1991; Thompson & Harrison, 1992; Brown *et al.*, 1993). The proportion of the inorganic mineral fraction is related to algal class. Diatoms with their silicious cell wall traditionally have a greater mineral content (Millamena *et al.*, 1990) and reduced inherent organic content compared with phytoflagellates (Whyte, 1987). Brown *et al.* (1997), analysed the gross composition of seventeen microalgal cultures from a range of algal classes (Figure 1.1).

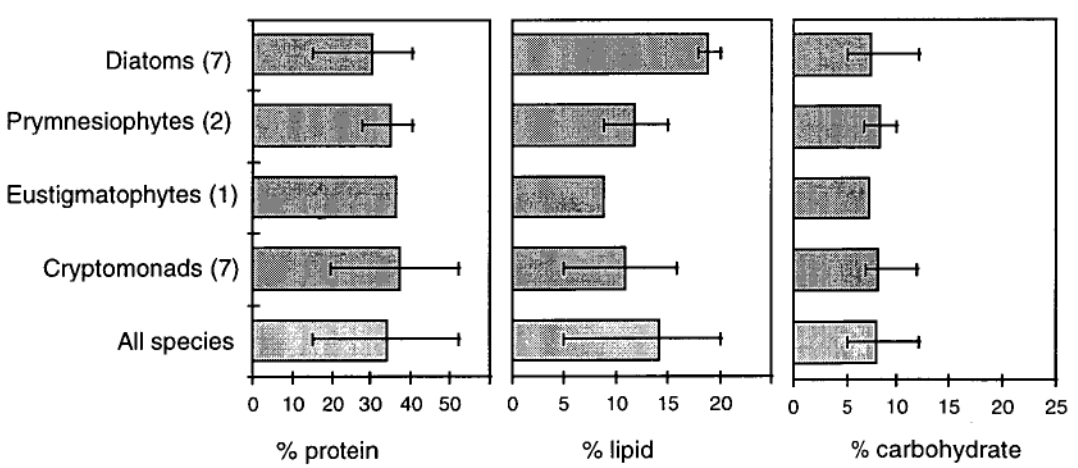


Figure 1.1 Percentage (DW) of protein, lipid and carbohydrate in microalgal cultures aerated with 1% CO_2. Range bars show the range of values. Reproduced from Brown *et al.*, (1997).

Their data shows that protein is the main constituent, followed by lipid then carbohydrate. Average protein levels of the algal classes were quite similar (30-40%)

but overall levels were variable, ranging from 15-52%. Lipid levels were variable between classes but diatoms tended to have the highest levels with least variation within the grouping. Carbohydrate levels were more consistent, ranging from 5-12%.

Gross compositions of algae are difficult to compare between different studies because they are so dependent on culture conditions. Brown *et al.* (1996), examined the effect of light intensity and photoperiod on the gross composition of *Thalassiosira pseudonana* harvested in logarithmic and stationary phase (Figure 1.2). They found that the percentage of protein decreased as cells entered stationary phase and that carbohydrate levels were most effected by light levels. Cells grown under 12:12 h light:dark contained less than half of the carbohydrate of 24:0 h light:dark grown cells.

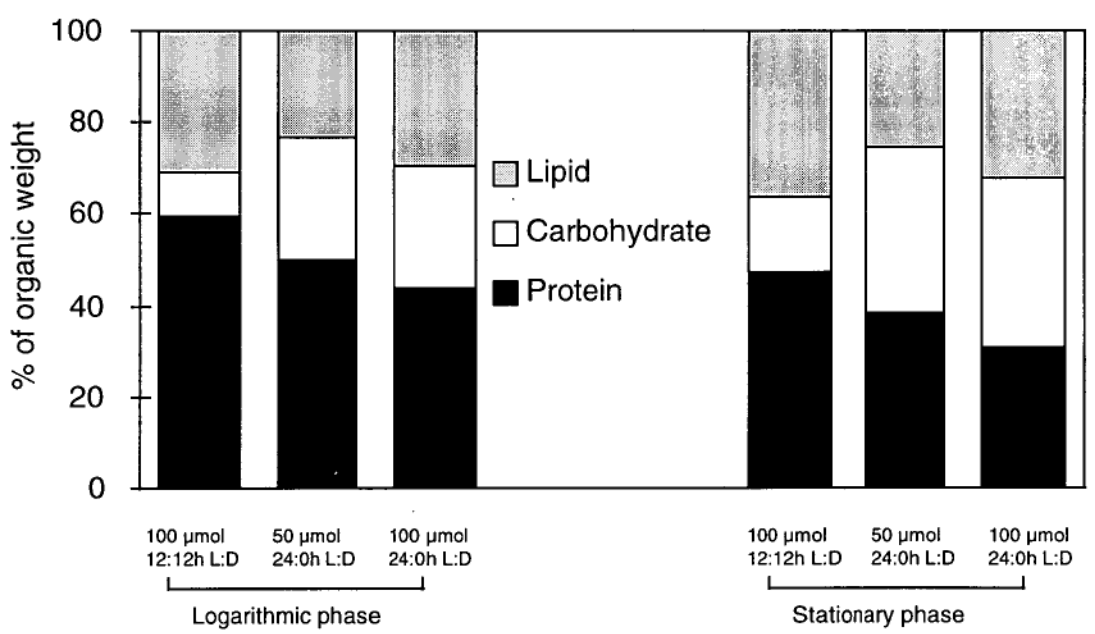


Figure 1.2 Gross composition of cultures of *Thalassiosira pseudonana* grown under different light regimes and harvested at logarithmic and stationary phase, expressed as % of organic weight. Reproduced from Brown *et al.*, (1996). µmol photon·m^{-2}·s^{-1} is abbreviated as µmol.

Harrison *et al.* (1990), found nitrogen limitation resulted in major changes to the composition of three microalgae (Figure 1.3). The percentage lipid in all species remained relatively constant, while the percentage of carbohydrate increased with a

corresponding reduction in protein. They also examined the effects of light limitation, but found it was not as dramatic or consistent as for nutrient limitation.

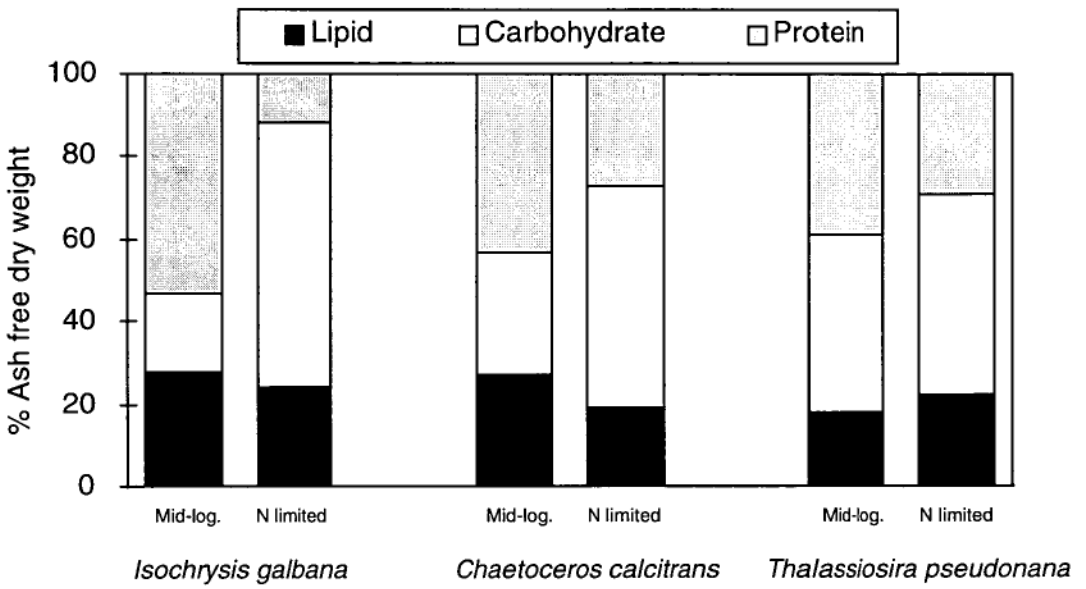


Figure 1.3 Gross composition of cultures of *Isochrysis galbana, Chaetoceros calcitrans* and *Thalassiosira pseudonana* harvested in mid-logarithmic phase and after nitrogen limited, expressed as % ash free dry weight. Reproduced from Harrison *et al.*, (1990).

Carbon and nitrogen are major elemental requirements of all aquacultured animal species. The relationship between cell volume and carbon and nitrogen content of phytoplankton populations has been used as an indication of nutritional value (Booth *et al.*, 1988). However, Verity *et al.* (1992), found that current regression models substantially underestimate the carbon density of nondiatomaceous marine nanophytoplankton. These small algal cells contain more carbon and nitrogen per unit volume than their larger phytoplankton members, mainly because they lack any significant vacuolar space.

1.2 ii. Nutritional Value to Molluscs

Aquaculture species are poikilotherms; therefore they do not need to expend energy as heat to maintain body processes. In fish <3% of metabolizable energy in a complete diet

is lost as heat (Smith *et al.*, 1978), making them the most efficient animals at converting feed into high quality protein.

The food value of different algae will depend on the specific nutritional requirements of the consumer species. Factors such as the animal's requirement for essential amino acids and lipids, algal digestibility, toxicity and calorific value, and its stage of growth will all determine the nutritional value of the algae. These factors vary between consumers so it is unlikely that any one alga will satisfy the nutritional requirements of all aquaculture species. Whyte (1987), recognised that these factors affect the ultimate food value of a microalgae. However, he suggests that the total calorific value of algae can be used as an indication of their nutritional value when the consumer species requirements are ill defined.

Ingestion rate also needs to be considered when assessing the food value of algae. Barillé *et al.* (1993), found that the smallest particle cleared by *Crassostrea gigas* with 100% efficiency rose from 4 to 8 μm as the seston load increased. They concluded that C. *gigas* did not adjust retention efficiency according to food quality but that gill porosity was determined by the concentration of total particulate matter. The oyster was able to regulate actual consumption through control of ingestion by production of pseudofaeces.

Ingested algal protein provides the consumer with their main source of nitrogen and amino acids. The protein requirement of molluscs is not well defined and Enright *et al.* (1986a), found no clear trend between protein levels present in microalgae and the growth response of the oyster *Ostrea edulis*. However, a protein content $<8.8\ \mu g \cdot 10^6$ cells^{-1} appears to restrict juvenile growth rate (Enright *et al.*, 1986b). Kreeger and Langdon (1993), fed juvenile mussels (*M. trossulus*) diets of algae with high or low protein levels and supplemented diet protein levels with microcapsules containing crab protein. Using this approach they found a dietary protein level below 40 % w/w and a C/N ratio above 10 limited the growth rate.

Algal carbohydrate is an important nutrient for animals feeding on them because it provides the main source of metabolic energy. In marine bivalves carbohydrate is rapidly metabolised, being preferentially catabolised as a respiratory substrate in both juveniles and adults (Thompson and Harrison, 1992). Manipulation of algae to produce feed with high levels of carbohydrate has been shown to improve growth in juvenile *Ostrea edulis*. However, high carbohydrate diets are only of nutritional benefit if sufficient levels of protein and lipids and essential PUFAs are also present (Enright *et al.*, 1986b).

The lipid fraction includes a wide range of energy rich compounds but Langdon and Waldock (1981), found that growth of *Crassostrea gigas* spat was not correlated with the total lipid content of the algae. Increasing the lipid content of the diet with microcapsules of triolein did not enhance growth of spat fed *T. suecica* or *D. tertiolecta.* However, microcapsules containing lipids rich in polyunsaturated fatty acids did increase growth.

Though it is generally well accepted that the gross composition of microalgae influences their nutritional value (Webb and Chu, 1983; Brown et al,. 1989), a direct correlation has been difficult to prove. For example, Thompson *et al.* (1993), found no apparent relationship between the gross composition of phytoplankton cells and their nutritional value for *Crassostrea gigas* larvae and Langdon and Waldock (1981), found that growth of *C. gigas* spat was not correlated with the total lipid content of the algae. The main problem evaluating algal diets based on their gross composition is that the three major organic fractions are inextricably linked and altering one by manipulating growth conditions will also effect the other fractions. The gross composition of the algae can also influence the consumers nutritional requirements with high levels of carbohydrate and lipids reducing demand for protein as a source of metabolic energy (Enright *et al.*, 1986b; Whyte *et al.*, 1989) and similarly excess protein can reduce carbohydrate demand.

Gross analysis is therefore not a sole indication of nutritional value and as discussed briefly in relation to lipids, composition of each fraction can be more important than

gross value. A more detailed examination of individual fractions is required for accurate assessment. In the following sections the organic fractions will be examined regarding their essential nutritional components in relation to molluscs.

1.3. Essential Components

1.3 i. Protein and Amino Acids

Phytoplankton are typically composed of over 40% protein (Pillay, 1990), but members of the Bacillariophyceae may have lower levels due to their significant silicious cell wall. The diatom *Chaetoceros calcitrans* has reported protein levels of between 30-35% DW (Brown, 1991; Utting, 1986) but levels as low as 25% were reported by Millamena *et al.* (1990b).

For algal consumers, protein is the major source of amino acids but an intracellular pool of free amino acids may constitute a significant proportion of the total available amino acids. In nitrate-grown *Isochrysis galbana* they equalled 1.5% of total cellular N and in ammonium-grown *Thalassiosira nordenskioldii*, 26.4% (Dortch *et al.*, 1984). Intracellular free amino acids are not constant and reflect the current and recent nutritional status of the algal cell (Flynn, 1990). Absolute amounts are even variable between culture batches, making exact determination of their nutritional value impossible (Flynn & Al-Amoundi, 1988). Of the methods used for protein analysis; dye-binding assays do not measure them as part of the total protein. However, summation of the anhydroamino acid residues gives a value which includes cellular protein and free amino acids and may be a more relevant method to assess the nutritional value of algae (Brown, 1991).

Consumer protein requirements are influenced by factors including animal species, genetic make-up, animal size, environmental factors (eg, temperature) and the amino acid composition of the protein. Of the 23 amino acids isolated from natural protein, ten are considered essential for fish, crustaceans and molluscs. These are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and for molluscs, proline may also be essential (Harrison, 1975).

Comparison of protein levels between publications is often complicated by variations in analytical and culture methods. Brown (1991), analysed the amino acid profiles of 16 species from six microalgal classes under conditions that minimised experimental

variations. He found the amino acid composition to vary little across the 16 species of microalgae, the minimum value for each amino acid seldom being less the half the maximum value. Comparing the amino acid profile of oyster larvae to the algae he concluded that essential amino acids are well supplied by the algae, with the possible exception of lysine. Kreeger and Langdon (1993), also found a higher percentage level of lysine in mussel flesh than in the algal protein (Table 1.3).

Table 1.3 Amino acid composition (weight percentage of total amino acids) of hydrolysates from 16 microalgae belonging to the Bacillariophyceae, Chlorophyceae, Cryptophyceae, Eustigmatophyceae, Prasinophyceae, Prymnesiophyceae and from oyster larvae. Data summarised from Brown, 1991.

	Algal Species						
	Prymnesiophyceae		Prasinophyceae				
	Bacillariophyceae		Cryptophyceae			Oyster larvae	Mussels **
Amino acid	*Isochrysis sp. (T.ISO)*	*Chaetoceros calcitrans*	*Tetraselmis suecica*	*Rhodomonas salina*	Range in 16 species	*C. gigas*	*M. trossulus*
Essential							
Arginine	7.4	6.4	13.2	6.7	5.9 - 13.5	7.5	5.11
Histidine	2	1.9	1.8	1.8	1.4 - 2.4	1.7	1.74
Isoleucine	4.6	5.5	3.5	4.1	3.4 - 5.8	4.4	4.17
Leucine	8.7	8.2	8	7.8	7.2 - 9.0	6.9	6.98
Lysine	6	6.3	6	6.1	5.1 - 6.3	8.2	7.82
Methionine	2.6	2.6	2.3	2.4	1.4 - 3.2	1.3	2.01
Phenylalanine	6.2	6.7	5.9	5.5	5.4 - 7.1	5.2	3.32
Proline *	5.9	5.6	4.7	5.4	4.6 - 13.1	4.4	4.20
Threonine	4.5	4.5	4.1	5.4	4.0 - 5.9	5.1	8.16
Tryptophan	1.6	1.4	1.2	1.3	0.86 - 1.6	1.6	0.47
Valine	6.1	5.9	5.7	6.1	5.7 - 6.7	5.4	4.30
Non-Essential							
Alanine	7.7	7.2	6.9	7.9	6.8 - 8.3	5.6	5.81
Aspartate	8.6	9.8	8.9	9.5	7.6 - 10.1	10.0	15.7
Cysteine	0.47	0.42	0.65	0.63	0.38 - 0.65	0.72	1.39
Glutamate	10.6	10.5	11.2	10.9	9.4 - 12.4	12.2	11.8
Glycine	6	5.9	5.9	5.8	5.1 - 6.4	9.1	5.68
H-proline	1	0.18	0.18	0.04	0.04 - 1.4	0.14	
Ornithine	0.38	0.21	0.8	0.28	0.21 - 1.4	0.64	
Serine	4.6	5.8	4.6	5.9	4.3 - 6.8	5.0	8.53
Tyrosine	4.3	4.5	3.8	5.6	3.2 - 5.6	4.5	2.85

* Essential for molluscs
** Kreeger and Langdon, (1993)

Generally, the amino acid profiles of phytoplankton have been found to vary little in composition between algal species or classes (Enright *et al.*, 1986a; Brown, 1991) or culture conditions (Brown *et al.*, 1993). However, culture temperature was found to effect levels of amino acids (James *et al.*, 1989). This could indicate that the majority of proteins perform similar functions in the different algae such as structural proteins.

Gross protein levels, like other cell constituents, are affected by culture conditions, but are reported to be largely independent of irradiance levels (Thompson *et al.*, 1993). However, nutrient limitation can result in a reduction of 60 % in protein content for *Chaetoceros gracilis*, even though the amino acid profile of remaining protein is unchanged (Enright *et al.*, 1986b).

Brown and Jeffrey (1992), used the essential amino acid index (EAAI) to evaluate the nutritional value of algal protein to oyster larvae. An EAAI value above 90 indicates a high quality protein, above 80 a moderate quality protein and below 70 an inadequate protein. They found that for ten species of green microalgae the EAAI equated to moderate to high protein quality. Because algal protein is a good source of essential amino acids for bivalve consumers it is generally not considered to be a limiting. Levels appear to be less critical than other macronutrients such as lipids (Enright *et al.*, 1986a).

1.3 ii. Carbohydrate

Carbohydrates form the primary food reserve in most algae with lipids also contributing a significant proportion in some classes. Factors leading to senescence or excess carbon fixation can result in increases in reserves of carbohydrates. Chu *et al.* (1982), analysed the carbohydrate composition of five microalgae used as feed for larvae of *Crassostrea virginica.* The principle sugars found were glucose, mannose, ribose/xylose, rhamnose and fucose with glucose accounting for between 28% and 86% of the total carbohydrate and mannose being a major sugar in diatoms.

Nutrient limitation and high light exposure have both been shown to significantly increase carbohydrate content (Enright *et al.*, 1986b; Fabregas *et al.*, 1986; Harrison *et al.*, 1990). Increases due to high light exposure are species-specific. Thompson *et al.* (1993), found no significant increase in carbohydrate or total carbon content over the light range 11-225 μmol photons$\cdot m^{-2} \cdot s^{-1}$ for three microalgae routinely used in oyster feed. Increases in carbohydrate level, due to nutrient limitation is a more consistent effect but varies between algae and the limiting nutrient. Enright *et al.* (1986b), reported a three-fold increase in the carbohydrate content of N-limited *Chaetoceros gracilis* cells. However, Whyte (1987), found the polysaccharide content in tested algae to be

similar for exponential and stationary phase cells except for *Thalassiosira pseudonana*. This diatom lost most of its polysaccharide content in stationary phase, which was attributed to the rapid utilisation of glucan for energy by this alga.

The carbohydrate fraction consists primarily of polysaccharide, typically 90-95% but a little lower for diatoms (~80%) (Brown, 1991). The remainder are mono-, di- and oligosaccharides. The primary storage polysaccharide of most microalgae are starches or chrysolaminaran (β-glucan) as in the case of the diatoms (Whyte, 1987).

Gross carbohydrate levels for microalgae have been regularly reported in the literature but few detailed biochemical analyses exist and variations in culture conditions make comparisons of limited significance. Brown (1991) conducted a comprehensive analysis of 16 microalgae from 6 classes that were cultured under conditions that minimised experimental variation and the polysaccharide (ie, the principal carbohydrate component) analysed for component sugars. Glucose was the predominant sugar, making up 21.0 - 87.5% of the polysaccharide (Table 1.4). Only in *Phaeodactylum tricornutum* was glucose exceeded by another sugar; mannose.

Brown's data and that of Chu *et al.* (1982), showed component sugars to vary greatly between and within algal classes. The two prasinophytes had very similar sugar profiles but profiles were variable between diatoms, chlorophytes and prymnesiophytes. Prasinophytes lacked xylose; eustigmatophytes lacked arabinose and the cryptophytes lacked rhamnose but these sugars may be present in other species from within these genera, not analysed in this study. There is little chance of using component sugar profiles as an aid to algal classification or as generalisations of algal class nutritional value. However, specific statements on nutritional value may be possible. Brown 1991, found the diatom *P. tricornutum*, had a high percentage of mannose (~ 46%) and proposed that this may help explain its low food value.

Table 1.4 Sugar composition ranges (weight percentage) of polysaccharide from algae belonging to the Bacillariophyceae, Chlorophyceae, Cryptophyceae, Eustigmatophyceae, Prasinophyceae and Prymnesiophyceae. Summary of data from Brown (1991).

Sugar composition ranges (weight percentage) of polysaccharide						
	Bacillariophyceae	Chlorophyceae	Cryptophyceae	Eustigmatophyceae	Prasinophyceae	Prymnesiophyceae
Sugar	6 species	2 species			2 species	4 species
Arabinose	0.15 - 2.4	0.65 - 0.16	0.08	0	0.41 - 0.90	1.6 - 11.7
Fucose	2.8 - 14.3	0.00 - 7.9	3	4.4	0	0.51 - 3.6
Galactose	5.4 - 20.5	1.1 - 10.6	2.7	3.8	11.3 - 15.7	4.4 - 19.0
Glucose	21.0 - 82.4	55.2 - 85.3	87.5	68.2	74.8 - 84.7	42.6 - 81.0
Mannose	2.0 - 45.9	4.5 - 5.0	2.3	6.1	1.8 - 3.0	3.6 - 13.2
Rhamnose	1.3 - 8.6	5.5 - 13.3	0	8.3	0.04 - 0.97	0.00 - 2.1
Ribose	1.4 - 5.4	1.0 - 2.0	2.5	4.6	1.8 - 4.5	2.0 - 3.8
Xylose	1.6 - 7.5	1.0 - 6.6	1.6	4.4	0	2.3 - 10.3

For consumers carbohydrates are not classified as essential or non-essential but for them to be of nutritional value the aquaculture species must have appropriate enzymes for their metabolism. In herbivores, amylase occurs throughout the digestive tract and most fish also have amylase activity although few have enzymes for cellulose metabolism (Pillay, 1990). Cellulose is digested by their gut microflora but its energy value may be lost or only partially available to the host. Cellobiase and chitobiase have been found in *Crassostrea virginica*, indicating oysters are well adapted for the complete digestion of chemically stable polymers (Mayasich and Smucker, 1986). Brock (1989), analysed hepatopancreas-cellulase activity in *Crassostrea gigas* fed *Tetraselmis suecica*. Oysters were either conditioned by prior feeding with cellulose rich diets or starved. Extracts from oysters previously fed cellulose-rich diets were better able to digest *T. suecica* than unfed oysters. There was no significant difference in the numbers of bacteria associated with sections of hepatopancreas tissue from the two trial groups. However, it was not possible to differentiate between algal cell lysis caused by hepatopancreas extract, from that caused by bacterial cellulase. The study was therefore unable to conclude that cellulase was of oyster origin.

1.3 iii. Lipids

The algal lipid fraction is a heterogenous class of compounds, broadly classified into two categories; polar and neutral lipids. The polar lipids include phospholipids and glycolipids that are both frequently associated with the structural components of the cell, most importantly cell membranes. Polar lipids are the major lipid fraction in actively growing algal cells, comprising from 92-99% of the extractable lipids in algae of the Chlorophyceae and Prasinophyceae classes (Dunstan *et al.*, 1992), and 83-90% in Prymnesiophytes and Eustigmatophytes (Dunstan *et al.*, 1993). The neutral or non-polar lipids are predominantly made up of tri- and diacylglycerols with alkenones, sterols, hydrocarbons, free fatty acids and pigments as minor constituents. These lipids perform a variety of functions of which energy storage is a primary role.

The distribution of lipids amongst the different lipid classes is dependent on culture temperature, light intensity, growth phase, nutrient status and in the case of heterotrophically grown algae, on C/N ratio and aeration (James *et al.*, 1989; Chen and Johns 1991; Hodgson *et al.*, 1991; Thompson and Harrison, 1992; Dunstan *et al.*, 1993). The proportions of major lipid classes vary between algae and also change as cultures enter stationary phase, a major factor determining the lipid distribution pattern (Table 1.5).

During stationary phase the level of polar lipids typically decrease significantly relative to the non-polar lipids. Triacylglycerols increase the most; they are often almost absent in logarithmic growth but constitute 20-41% of total extractable lipids in the stationary phase. Some diatoms and cryptomonads have significant levels of triacylglycerols and free fatty acids during exponential growth, demonstrating the difficulty in making general statements about microalgae. The decrease in polar lipids and corresponding increase in non-polar lipids during stationary phase is a reflection of the metabolic status of the cell. During exponential growth polar lipids are required for maintenance of and synthesis of new structural components such as cell membranes. However, during stationary phase cell division almost ceases and excess energy is directed toward synthesis of energy rich storage compounds such as triacylglycerols.

Table 1.5 Proportions of major solvent-extractable lipid classes in batch cultures of aquaculture species of microalgae. * Data extracted from Dunstan *et al.*, (1993). ** Data extracted from Volkman *et al.*, (1989).

Algal culture	Phase	Day	Lipid class (%)								
			PL	SD	ST	4ME	FFA	TG	HC	MK	EK
*Nannochloropsis oculata**	Logarithmic	9	90		4		2.4	1.9	1		
	Stationary	23	51		5		0.5	41	3		
Pavlova lutheri *	Logarithmic	9	88	5	3	1.4	3.1	tr			
	Stationary	23	57	6	5	2.5	1.2	28	1		
Isochrysis sp. *	Logarithmic	9	83		1			0.6		11	3.8
	Stationary	23	54		1			20		22	4.1
*Chaetoceros calcitrans***	Logarithmic		73		6		11	8.4	0		
Chaetoceros muelleri **	Logarithmic		44		6		14	34	1		
Tetraselmis suecica **	Logarithmic		92		2		0.8	3.3	2		
Rhodomonas salina **	Logarithmic		68		5		1.9	22	4		

PL, polar lipids and chlorophylls; SD, steroidal diols (tentative identification); ST, 4-desmethyl sterols; 4ME, 4-methyl sterols; FFA, free fatty acids; TG, triacylglycerols; HC, hydrocarbons; MK, C_{37} - C_{38} methyl ketones; EK, C_{38} - C_{39} ethyl ketones. tr= trace, < 0.5%.

1.3 iv. Fatty acids

Fatty acids are the dominant component of the lipid fraction, comprising 20-40% of the total extractable lipid by weight (Cohen, 1986). They generally occur in an esterified form with glycerol (Figure 1.4) and belong to both the polar and non-polar lipid divisions.

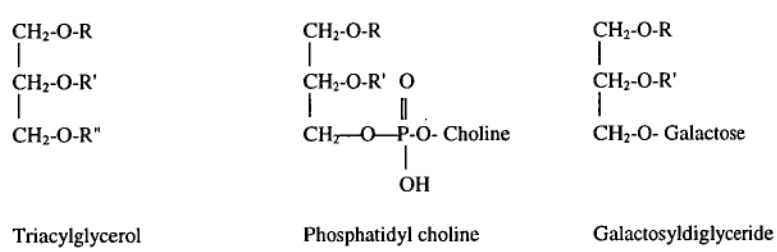


Figure 1.4 Structure of lipid components of algae. R, R' and R" represent fatty acid groups.

Fatty acids can include such short chain compounds as acetic, propionic and butyric acids, though it is the longer carbon chain molecules from C_{12} to C_{22} that are referred

to as fatty acids when analysed in the extractable lipid fraction. Fatty acids are typically straight chains of an even number of carbon atoms but fatty acids with 15, 17, and 19 carbon atoms are detected in some algae (James *et al.*, 1989; Dunstan *et al.*, 1992, 1993).

The chemical properties of fatty acids reflect the reactivity of the carboxyl group, other functional groups and the degree of unsaturation in the carbon chain. Their melting points increase with increasing chain length but this effect is reduced as the degree of unsaturation increases. This is an important property for maintaining fluidity in cell membranes at low temperatures. Fatty acids containing multiple double bonds are referred to as polyunsaturated fatty acids (PUFAs), as unsaturation increases some authors then refer to these as highly unsaturated fatty acids (HUFAs).

The fatty acid composition of microalgae commonly used in aquaculture varies considerably between algae and algal classes (Table 1.5). There is similarity between some algae within a class but other members show marked differences. It is therefore not possible to make finite statements characterising fatty acid profiles and algal class, only trends. Diatoms and prymnesiophytes tend to have higher proportions of saturated and monounsaturated fatty acids than the green algae and cryptophytes. The cryptophyte, *R. salina* has very low levels of monounsaturates (7.9%) with no detectable C_{16} polyunsaturates but high levels of polyunsaturates C_{18}, C_{20} and C_{22}. This alga had the highest total percentage of polyunsaturates, approaching 70% of extractable lipid. The diatom *T. pseudonana* has an unusual profile compared to other diatoms. It has lower levels of saturated and monounsaturated fatty acids and a higher level of polyunsaturates, notably 20:5n-3, eicosapentaenoic acid (EPA) and 22:6n-3, docosahexaenoic acid (DHA). *Dunaliella tertiolecta* lacks these two n-3 PUFAs which are generally recognised as being deficient in chlorophytes (Dunstan *et al.*, 1992). However, very low proportions of C_{20} and C_{22} PUFAs have been noted for a few marine chlorophytes (James *et al.*, 1989; Volkman *et al.*, 1989).

Table 1.5 Fatty acid composition of microalgae used in aquaculture grown under similar conditions and harvested in late-logarithmic phase. Data extracted from Volkman *et al.* (1989).

	Algal Species							
	Prymnesiophyceae		Bacillariophyceae			Prasinophyceae	Chlorophyceae	Cryptophyceae
Fatty acid	*Isochrysis* sp (T.ISO)	*Pavlova lutheri*	*Chaetoceros calcitrans*	*Chaetoceros muelleri*	*Thalassiosira pseudonana*	*Tetraselmis suecica*	*Dunaliella tertiolecta*	*Rhodomonas salina*
Saturates								
Total Sum %	32.2	35.9	30.2	35.5	27.2	26.8	15.4	22.2
Monounsaturates								
Total Sum %	26.1	20.4	33.8	38.5	19.5	20.5	5.2	7.9
Polyunsaturates								
Sum C 16	2.6	0.8	13.4	5.3	22.2	16.2	25.9	0.0
18 : 2 (n-6)	2.5	1.5	0.8	0.7	0.4	13.9	4.8	10.5
18 : 3 (n-6)	2.4	0.4	0.4	1.1	0.2	2.7	2.7	2.6
18 : 3 (n-3)	3.6	1.8	TR	-	0.1	4.6	43.5	14.2
18 : 4 (n-3)	17.4	6	0.5	1.2	5.3	4.8	1.0	21.3
20 : 4 (n-6)	-	TR	5.7	6.2	0.3	2.1	-	0.9
20 : 4 (n-3)	-	-	0.2	TR	0.3	0.1	-	1.0
20 : 5 (n-3)	0.2	19.7	11.1	5.7	19.3	5.3	-	11.9
22 : 5 (n-6)	1.8	2	-	-	-	-	-	0.3
22 : 6 (n-3)	8.3	9.4	0.8	0.4	3.9	TR	-	5.2
Total Sum %	41.3	42	33.7	24.8	52.6	49.7	77.9	67.9
Others	0.5	1.8	2.3	1.2	1.3	3	1.5	2.0

TR = Trace amount

The distribution between the types of fatty acids are not static and major differences can occur as cells enter stationary phase (Dunstan *et al.*, 1993). Results published by Shamsudin (1992), show these changes to occur continually as a culture ages. The main observation was a continual increase in the relative proportion of saturated and monounsaturated fatty acids with a corresponding fall in polyunsaturates. Fatty acid profiles will therefore depend on whether cells are examined during early-, mid-, late-logarithmic or stationary phase growth.

Considerable recent research has focused on the role fatty acids play in animal nutrition, particularly the role of polyunsaturated fatty acids (PUFAs). The C_{20} n-3 and n-6 fatty acids have been shown to be essential to several aquaculture species including oysters (Langdon and Waldock, 1981; Enright *et al.*, 1986a,b). In the rock scallop, *Crassadoma*

gigantea (Gray) the fatty acids 16:0, 18:0, 18:1n-7, 20:5n-3 and 22:6n-3 are accumulated by larvae, indicating a possible requirement for these acids during the larval phase (Whyte *et al.*, 1990).

Enright *et al.* (1986a), analysed the nutritive value of sixteen phytoplankton species for juvenile *Ostrea edulis*. Five out of the top six algal species were diatoms, the other a cryptomonad. The diatom species tend to have high levels of saturated fatty acids, good sources of metabolic energy and a moderate to good supply of one or both of the essential PUFAs, 20:5n-3 and 22:6n-3. *T. pseudonana* has lower levels of saturated and monounsaturated fatty acids but very high levels of both 20:5n-3 and 22:6n-3 (Volkman *et al.*, 1989). The cryptomonad *Rhodomonas* sp., found to be a good diet for juvenile *Ostrea edulis* source by Enright *et al.* (1986a), had high levels of protein, carbohydrate and the essential PUFA, 22:6n-3. Another cryptomonad, *Rhodomonas salina* (Table 1.5) also has high levels of both essential PUFAs, 20:5n-3 and 22:6n-3 while having relatively low proportions of saturated and monounsaturated fatty acids.

The prymnesiophytes are popular mariculture species, particularly *Isochrysis* sp. (T-iso) and *Pavlova lutheri* which both have high proportions (8.3 and 9.4% total fatty acids) of 22:6n-3; *P. lutheri* also has a major proportion of 20:5n-3 (19.7% of total fatty acids) (Volkman *et al.*, 1989). For juvenile oysters, *I. galbana* (T-iso) is the most frequently cultivated microalgae (Coutteau and Sorgeloos, 1992), even though it is outperformed by several diatom species. Although a good aquaculture species, the lower performance of T-iso has been attributed to its low carbohydrate level (Enright *et al.*, 1986a).

1.3 v. Phospholipids

Phospholipids are considered to be derivatives of phosphatidic acid, containing a phosphorous atom in addition to glycerol, fatty acids and a nitrogenous base. They are widespread across animals, plants and bacteria with a high degree of similarity of structure and are associated with membranes. They are amphipathic compounds possessing both polar and non-polar functions.

Few studies have examined the different phospholipid classes of microalgae. Ben-Amotz *et al.* (1985), reported the major algal phospholipids to be phosphatidyl inositol, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine and diphosphatidyl glycerol. They reported levels to vary between 5-25% of the total lipid fraction with an average of 10%.

Phosphatidyl choline (lecithin) is sometimes added to aquaculture diets although its precise role in nutrition is unclear. It is thought to play a role in the transport of lipids in crustacean haemolymph (Pillay, 1990). Crustaceans are able to synthesise phospholipids but not fast enough to satisfy their metabolic demands (Ewing and Finmore, 1970). Dietary requirements for molluscs are not defined but the phospholipid content of oysters remain almost constant during larval development, metamorphosis and early spat growth with an average level of 7.7% of the total organic matter (Holland and Spencer, 1973).

1.3 vi. Sterols

Sterols form part of the neutral lipid fraction and are planer, rigid, hydrophobic, multicyclic molecules. They act as structural components in cell membranes and as effectors. As structural components they modulate membrane fluidity as a function of their concentration and buffer the effect of temperature on plasma membranes, maintaining their integrity. As effectors they are first converted to end products such as steroid hormones or bile components. They can also bind to membrane proteins, modulating their activity (Patterson, 1991).

In plants there are relatively few (≈6) dominant sterols but algae contain a complex variety of dominant sterols (Patterson, 1991). Generally they are only present at low levels in mariculture microalgae, ranging from 0.2-6.3% of the total extractable lipid (Volkman *et al.*, 1989).

Cyanobacteria, originally thought to contain no or very limited sterols, have now been shown to contain a range of sterols even though they only comprise 0.005 - 0.03% dry weight (Patterson, 1991). The chlorophytes have the widest range of major sterols.

However, the more primitive orders including mariculture species contain only traces of sterols while the related prasinophytes contain up to 3% of extractable lipids as sterols (Dunstan *et al.*, 1992). Many chlorophytes contain ergosterol, considered to remain from ancient times when ozone levels were low and ultraviolet radiation high. Ergosterol present in cell membranes is an effective absorber of ultraviolet radiation (Patterson, 1991).

The diatoms, prymnesiophytes and cryptomonads tend to have the higher levels of sterols, mainly comprising cholesterol and 24-methyl and ethyl derivatives. Sterols of eustigmatophytes are mostly cholesterol (52-80%), with smaller amounts of 24-ethylcholesterol and iso-fucosterol (Patterson *et al.*, 1994). The dinophytes differ from other algal classes by the presence of unusual quantities of sterols containing the 4α methyl group and the presence of dinosterol (4α, 23,24 - trimethyl - 5α -cholest -22-en-3β-ol) (Patterson 1991).

The sterols present in molluscs vary between species with the oyster *Crassostrea virginica* capable of having over 45 sterols, reflecting the complexity of the ingested dietary sterols. Molluscs may have a limited capacity to synthesis sterols but little evidence exists and sterols are generally assumed to be an essential requirement although precise levels are not known. Knauer *et al.* (1998), showed that *C. gigas* spat is capable of both alkylation and dealkylation of dietary sterols. Growth of *C. virginica* spat was positively correlated with algal sterols (delta-5, except those with ethyl substitution on carbon-24) (Wikfors *et al.*, 1991).

1.3 vii. Hydrocarbons, Alkenones and Pigments

Hydrocarbons, alkenones and pigments all form part of the total lipid fraction of algae. Hydrocarbons and alkenones make up only a minor portion (0.1-0.2%) of the total lipid fraction (Brown *et al.*, 1989). Pigments include chlorophylls and the accessory pigments; carotenoids (carotenes and xanthophylls) and water soluble phycoerythrins and phycocyanins. The pigment β-carotene is also a hydrocarbon and a common carotenoid in plants. It is found in highest concentration in green algae and in

Dunaliella salina it may accumulate to up to 10% organic weight (Ben-Amotz and Avron, 1989) where it performs a photoprotective role.

Algal classes have defined profiles of pigments that can be used for identification (Jeffrey *et al.* 1997). Eustigmatophytes have been misidentified as xanthophytes or chlorophytes but they have a unique pigment profile. They are identified by the absence of chlorophyll b and c and the presence of violaxanthin and/or vaucherioxanthin as the main accessory pigments (Patterson *et al.*, 1994).

The role of chlorophylls in nutrition of animals is not known but it is likely that they supply a small amount of nitrogen and carbon from their digestion. Animals are not able to synthesise carotenoids although some are able to modify a limited selection (Estermann, 1994). β-carotene (pro-vitamin A) is a source of vitamin A, while xanthophylls are incorporated into the exoskeleton of crustaceans and the flesh and eggs of salmonoids (Cohen, 1986). These pigments are conserved during moulting and play a major role in pigmentation but other functions are yet to be defined.

1.3 viii. Vitamins

Vitamins are a group of chemically unrelated organic compounds required by organisms in varying numbers but only in minute quantities. They are usually classified as water-soluble and fat-soluble. The water-soluble vitamins include the eight members of the vitamin B complex: thiamine, riboflavin, pyridoxine, pantothenic acid, niacin, biotin, folic acid and vitamin B_{12}. They also include the essential nutritional factors choline, inositol, ascorbic acid and the less well defined vitamins ρ-aminobenzoic acid, lipoic acid and citrin. The fat-soluble vitamins include vitamins A, D, E (tocopherol) and K.

Algae can generally synthesise their required vitamins and are a good source of supply of these to other organisms. The production of vitamins, like many other cellular components, is dependent on cultivation conditions. Brown and Miller (1992), looked at the effects of light intensity and growth phase on the ascorbic acid (vitamin C) content of eleven species, covering all the aquaculture microalgal classes. They found ascorbic acid levels varied greatly between species with levels affected by light intensity

and growth phase, though not in a predictable way. Stimulation by light exposure could make heterotrophically grown algae a poorer source of vitamins than light cultured algae. In a study looking of the riboflavin content of six species of microalgae Brown and Farmer (1994), noted that the proportion of riboflavin increased in all species following the onset of stationary phase.

Seguineau *et al.* (1996), undertook a detailed analysis of vitamin profiles of three microalgae used to feed scallop larvae (Table 1.6). Comparing levels to those used in fish culture, they found microalgae to be a rich source of vitamins with the possible exceptions of pyridoxine, biotin and pantothenic acid. The vitamin requirement of bivalve molluscs is presently ill defined. The lack of a totally artificial diet and the unknown contribution made by extracellular algal vitamins complicate efforts to determine requirements. Also, additional sources from ingested bacteria and the existence of symbiotic relationships in some bivalves further complicate quantification of vitamins and other nutrients. Seguineau *et al.* (1996), isolated bacteria from scallop larvae rearing tanks and found some strains to be rich in pantothenic acid, with similar levels of other vitamins to those of microalgae. They determined that bacteria contribute 81% of the pantothenic acid and 32% of biotin and folic acid available to scallop larvae in the rearing tanks.

Table 1.6 Mean vitamin composition of three species of microalgae, *Pavlova lutheri*, *Isochrysis galbana* (T. iso) and *Skeletonema costatum*. Table reproduced from Seguineau *et al.*, (1996).

	Average vitamin = $\mu g \cdot g^{-1}$ dry weight		
Vitamin	*P. lutheri*	*I. galbana* (T. iso)	*S. costatum*
Ascorbic acid (C)	563	1926	1457
Thiamine (B_1)	42	40	113
Riboflavin (B_2)	30	38	28
Pyridoxine (B_6)	10	8.2	6.7
Biotin	1.02	0.7	0.71
Cyanocobalamin (B_{12})	1.79	7.37	3.91
Niacin (PP)	140	465	108
Folic acid	7.2	15	12.5
Pantothenic acid	25	38.2	13.7
α-Tocopherol (E)	346	170	159
β-Carotene	1205	426	490

Although microalgae are a good source of a broad range of vitamins (Brown and Miller, 1992; Seguineau *et al.*, 1996) their concentration can vary greatly depending on growth phase (Brown and Farmer, 1994). The chance of vitamin deficiency in animals would be minimised if fed on a mixed algal diet.

1.4 Substitutes for Live Algal Diets

While appropriately chosen microalgae offer excellent nutritional value for larval and juvenile animals, their production can constitute a major ongoing cost for hatcheries. As well as being more convenient than live microalgae, they could lower operating costs through more efficient use of resources. For this reason there has been research into the development of 'off-the-shelf' products that could replace the need to maintain live algal cultures. These have included; spray-dried heterotrophically-grown algae (Biedenbach *et al.*, 1990; Laing and Gil Verdugo, 1991; Laing and Millican, 1991, 1992), algal pastes and concentrates (Watson *et al.*, 1986; Donaldson, 1991; Nell and O'Connor, 1991; Brown, 1995), micro-encapsulated diets (Langdon and Bolton, 1984; Laing, 1987; Knauer and Southgate, 1997) and yeasts and bacteria (Urban and Langdon, 1984; Coutteau *et al.*, 1990; Nell *et al.*, 1996). In a review of substitutes for live microalgae in mariculture, Robert and Trintignac (1997), concluded that concentrated and dried microalgae are currently the best alternative feed products for bivalves.

Much of the published work on the production of algal concentrates and pastes has involved the use of centrifugation. Of the algae commonly used in aquaculture, diatoms have shown the most potential. Watson *et al.* (1986), found *Thalassiosira pseudonana* and *Chaetoceros calcitrans* withstood the centrifugation process well and the cells were easily resuspended. However, they found the nutritional value declined at an unpredictable rate when stored at 4°C. Donaldson 1991, also found that the nutritional value of centrifuged algal pastes fell rapidly on storage at 4°C with a shelf-life of only about 10 days. Nell and O'Connor (1991), fed six algal species, singly and in combination with *Pavlova lutheri*, in both fresh and concentrated form, to Sydney rock oyster (*Saccostrea commercialis*) larvae. They found, that when concentrated to a paste and stored for 7-14 days at 4°C, a combination of *P. lutheri* and *C. calcitrans* produced greater length increase than any other fresh or stored, single or combined diet tested. However, as a single diet the prymnesiophytes, *P. lutheri* and Tahitian *Isochrysis* produced significantly less growth than when fed as live algae. Watson *et al.* (1986) found the same species were severely damaged by centrifugation. Small, tough microalgae such as *Nannochloropsis* and *Chlorella* have been centrifuged to algal

pastes and successfully used as nutritional feeds in the culture of the rotifers *Brachionus plicatilis* (Lubzens *et al.*, 1995), and *B. rotundiformis* (Yoshimura *et al.*, 1996).

An alternative concentration method to centrifugation is flocculation with sedimentation or flotation. It is routinely used as a method to clarify water; removing suspended solids that include microalgae. A major application is in the clarification of drinking water with removal of algae which may include toxic species (Petrusevki et al., 1995; Vlaski et al., 1996). In this application the flocculated algae is a waste product. It is also used to clarify sewage treatment ponds (Viviers and Briers, 1982; Koopman and Lincoln, 1983; Buelna et al., 1990), where the flocculated algae may be used as a source of supplementary nitrogen for ruminants (Hasdai and Ben-Ghedalia, 1981). In the production of natural beta-carotene from *Dunaliella salina,* flocculation is used to harvest the mass cultures of algae (Ben-Amotz and Avron, 1989).

In the flocculation of freshwater microalgae, polymers (chitosan, and polyelectrolyte compounds derived from polyacrylamide), can be used as the sole flocculating agent (Lavoie and de-la-Nouee, 1983; Bilanovic *et al.*, 1988; Sukenik *et al.*, 1988; Buelna *et al.*, 1990). However, salinity inhibits flocculation due to the high ionic strength which interferes with electrostatic bonding between algae and polymer and also causes the long chain polymer to shrink, decreasing its ability to bridge between algal cells (Bilanovic *et al.*, 1988). Sukenik *et al.* (1988), found inhibition of flocculation was diminished at reduced salinity levels, with effective flocculation attained at a salinity level of 5 $g \cdot L^{-1}$. In marine systems polymer flocculants are often used in conjunction with inorganic coagulants (Fe^{+3}, alum, lime) where they improve flocculation efficiency (Adin and Klein-Banay, 1986; Sukenik *et al.*, 1988).

1.5 Thesis Outline

Two main areas of research were chosen for this thesis. They were: 1) isolation and characterisation of local Australian microalgae suitable for use in aquaculture (Chapter 3 and 4); 2) development of methods to concentrate microalgae and determination of their nutritional value as feeds for juvenile Pacific oysters *Crassostrea gigas* (Chapter 5 and 6). The topics are linked together by the unifying theme of improved algal feeds for aquaculture and the methods developed to concentrate microalgae are applicable for use with the isolated Australian species. The research has significance in that it broadens the range of nutritionally evaluated microalgae available for use in aquaculture and provides further insight into the qualities that make algae nutritious. In an expanding and increasingly competitive aquaculture industry, operators have experimented with alternatives to live algal feeds as a method to streamline their operations. However, only limited data is available on the effectiveness of these alternatives. This work provides significant results to researchers and industry on the nutritional value of algal pastes and concentrates and details methods for their production.

The following provides a brief chapter outline, introducing their contents and significance. Each chapter includes an introduction followed by materials and methods (specific to that chapter), results and discussion sections.

Chapter 1. Literature Review

The chapter provides a summary of research relating to algal composition and its relation to their nutritional value as aquaculture feeds, with an emphasis on the nutritional requirements of molluscs. It also provides a framework for the theme of the thesis and chapter discussions.

Chapter 2. General Material and Methods

Commonly used methods used throughout this thesis are detailed in this chapter. These techniques are placed together in order to avoid repetition in subsequent chapters.

Chapter 3. Isolation and Characterisation of Australian Microalgae

This chapter details the methods used to isolate microalgae, their purification and identification. Summaries of the biochemical analyses of isolates are presented and a complete data set is contained within the thesis as Appendix 1 and 2. Recommendations are made on the likely aquaculture potential of isolates based on their composition and growth characteristics.

Chapter 4. Assessment of Local Isolates as Oyster Feed

Isolates selected in Chapter 3 as having potential as feed sources for juvenile oysters are evaluated. They are fed to juvenile *Crassostrea gigas* as part of a ternary diet containing a minor prymnesiophyte component. Two isolates are shown to be excellent feed species and the differences in diet performance are discussed in relation to diet composition.

Chapter 5. Production of Algal Concentrates by Centrifugation

This chapter details the production of algal pastes by centrifugation. The suitability of centrifugation as a method to concentrate a range of microalgae is determined and the physical properties of the pastes are measured. Their nutritional value to juvenile Pacific oysters is evaluated and results are discussed in relation to nutritional deficiencies and their causes.

Chapter 6. Production of Algal Concentrates by Flocculation

Chapter 6 further develops production of algal concentrates, based on the findings of chapter 5. Alternative methods to concentrate microalgae based on flocculation are developed and their qualities are compared to those produced by centrifugation. A feeding experiment with juvenile Pacific oysters compares concentrates produced by centrifugation and flocculation to a live algae diet.

Chapter 7. General Discussion

This chapter summarises the significant findings made in the research chapters, it considers outstanding issues and assesses the aquaculture potential of the developed techniques.

Chapter Two

General Materials and Methods

Chapter 2

General Materials and Methods

Introduction

To prevent repetition and having to present lengthy materials and methods sections in individual chapters, the following commonly used techniques are described below.

2.1. Maintenance of Live Organisms

2.1 i. Algal Culture Conditions and Growth

A modified f/2 (Guillard and Ryther, 1962), enriched natural seawater media was used for isolation, maintenance and scale-up of algal isolates (Table 2.1). Algal stock cultures were maintained in 125 mL Erlenmeyer flasks containing 75 mL of f/2 media. All nutrients except phosphate were added to 0.2 µm filtered seawater (0.5 mL nutrient stock·L^{-1}) which was dispensed to flasks and autoclaved (121°C, 15 psi, 15 min). The phosphate stock was diluted with Milli-Q H_2O and sterilised separately to avoid formation of precipitate. It was later added to each sterilised flask to give a final concentration of 0.5 mL stock·L^{-1}.

For larger volumes of f/2 (1-10 L), filtered seawater was dispensed into culture vessels and autoclaved (121°C, 15 psi, 30-60 min). Stock solutions (including phosphate) were combined together with dilution (1:3) in Milli-Q H_2O, autoclaved, then added to the sterile seawater at the point of algal inoculation. For culture of diatoms in polycarbonate carboys (Nalgene), silica (f/2) was added to the filtered seawater before autoclaving. With addition of f/2 nutrients at the point of algal inoculation, the final silica concentration was F strength. Since no buffering agents were added, the pH of the sterile seawater was allowed to re-equilibrate over several days before use. When inoculated, cultures were mixed and aerated by bubbling a filtered 1% CO_2/air mix (20 L·h^{-1}).

Mass culture of algae was undertaken in polyethylene bags and open polyethylene or fibreglass tanks. Bags (100 and 500 L) and tanks (500-1000 L) were filled with 0.2 µm filtered seawater and fertilised by addition of diluted sterile nutrients (f/2). Sterile inoculum (1-20 L) was added and cultures mixed and aerated with a filtered 1% CO_2/air mix (50-100 $L \cdot h^{-1}$).

Solid media was prepared by adding agar (Oxoid No1) to filtered seawater. After autoclaving the agar media was mixed and sterile stock nutrients added. It was then poured into Petri dishes in a laminar flow cabinet. These were left to set with their lids off then re-assembled and stored in sealed bags at 4°C. When required, plates were allowed to reach room temperature and if too wet, dried in a laminar flow cabinet before being inoculated with algae.

Table 2.1 Modified (CSIRO) f/2 media. All reagents are analytical reagent (A.R.) grade. Vitamins (Sigma Chemicals); Thiamine HCl (T-4625), d-Biotin (B-4501), Vitamin B_{12} (V-2876).

Stock	Nutrient	Nutrient·L^{-1} H_2O	Working concentration (f/2)
1	$NaNO_3$	150.0 $g \cdot L^{-1}$	883 µM
2	$NaH_2PO_4.2H_2O$	11.3 $g \cdot L^{-1}$	36.2 µM
3	$CuSO_4.5H_2O$	19.6 $mg \cdot L^{-1}$	0.04 µM
	$ZnSO_4.7H_2O$	44.0 $mg \cdot L^{-1}$	0.08 µM
	$CoCl_2.6H_2O$	22.0 $mg \cdot L^{-1}$	0.05 µM
	$MnCl_2.4H_2O$	360.0 $mg \cdot L^{-1}$	0.91 µM
	$NaMoO_4.2H_2O$	12.6 $mg \cdot L^{-1}$	0.03 µM
4	$NaSiO_3.5H_2O$	22.7 $g \cdot L^{-1}$	60.0 µM
5	Ferric citrate	9.0 $g \cdot L^{-1}$	15.0 µM
	Citric acid	9.0 $g \cdot L^{-1}$	
6	Thiamine HCl	20.0 $mg \cdot L^{-1}$	0.1 $mg \cdot L^{-1}$
	Biotin	0.1 $mg \cdot L^{-1}$	0.5 $\mu g \cdot L^{-1}$
	Vitamin B_{12}	0.1 $mg \cdot L^{-1}$	0.5 $\mu g \cdot L^{-1}$
	f/2 = 0.5 mL of each stock per L seawater		

Algal stock cultures (75 mL) were maintained at 17.5°C on a 12:12 h light/dark cycle and illuminated from below by cool white fluorescent lights at 45 μmol photon·m^{-2}·sec^{-1} average light intensity. Cultures were subcultured every four weeks by inoculating a new flask with 1 mL of culture. The new daughter culture was maintained as outlined while its parent and grandparent cultures were kept under low light (10-20 μmol photon·m^{-2}·sec^{-1}) as back-up cultures. Axenic status was checked at point of subculture by placing a drop of culture on a seawater agar with vitamins (SWAV) plate (section 2.1 iii.). Plates were incubated (20 ± 2°C) for 7 days to confirm no bacterial contamination.

Algal stock cultures were used to inoculate 2 L Erlenmeyer flasks containing 1.5 L of f/2 media. These were maintained at 20°C with continuous illumination from below using cool white fluorescent lights at 120 μmol photon·m^{-2}·sec^{-1}. Polycarbonate carboys (10 L) were inoculated using 1.5 L cultures and maintained at 20°C with continuous illumination from below at 200 μmol photon·m^{-2}·sec^{-1}.

Algal growth rates were calculated during logarithmic growth by measuring the rate of increase in cell number. Cell numbers were measured using an improved Neubauer Haemocytometer (American Optical). Motile species of algae were killed by addition of a few drops of lugols solution prior to counting (Throndsen 1978). Instantaneous growth rate (μ; d^{-1}) was calculated as:

$$\mu = (\ln N_1 - \ln N_0) / (t_1 - t_0)$$

where, N_1 = measurement at time 1 (t_1), and N_0 = measurement at time 0 (t_0). Growth rate (divisions·d^{-1}) was calculated as:

$$\text{divisions}\cdot d^{-1} = \mu / \ln 2$$

Cell counts were inaccurate for isolates with strong clumping tendencies (*Nitzschia* cf. *paleacea* isolates CS-430, CS-433); for these, DW were substituted for cell number as an estimation of growth. For algal dry weights (DW), a known volume (eg. 50-100 mL) of culture was filtered under vacuum onto a pre-weighed, pre-combusted glass fibre filter paper (4.7 cm Whatman GF/C). For algal pastes and concentrates a measured aliquot was resuspended in filtered seawater and a known volume filtered. All filters were rinsed (Brown 1991) with 30 mL 0.5 M ammonium formate ($CH_2O_2.NH_3$) and

dried overnight at 100°C then re-weighed. To calculate ash free dry weights (AFDW) the dried filters were combusted overnight at 450°C and re-weighed. Ash and AFDW was calculated by subtraction of the ashed filter weight from the DW measurement.

2.1 ii. Juvenile Oyster Culture and Feeding Experiments

Live algae and algal concentrates were assessed for their nutritional value as diets for Pacific oyster spat (*Crassostrea gigas* Thunberg). Juvenile oysters were provided by Shellfish Culture Ltd. and feeding experiments conducted at their nursery at Pipe Clay Lagoon, 30 km Southeast of Hobart, Australia (42° 58' S, 147° 32' E). For oyster culture, scaled down ($1/20^{th}$) models of commercial upwellers were used (Rodhouse *et al.*, 1981). They consisted of a 10 L bucket with a 110 mm diameter mesh-bottomed chamber suspended inside (Figure 2.1). Each bucket contained a submersible aquarium pump (Aquarium Powerhead 480) to circulate water through the chamber. Culture buckets were maintained in a temperature-controlled room (18 ± 2°C) and filled with 8 L of filtered (0.6 μm), temperature-equilibrated seawater. Buckets were cleaned daily and refilled with filtered seawater. Chambers were cleaned daily with a fine spray of filtered seawater to remove fouling from the mesh. Freshly graded oysters (700-1500 μm) were added to each chamber (1 mL packed volume) and evenly distributed over the mesh screen. Oysters were allowed to acclimatise for two days before feeding. Four, randomly distributed replicates of each test diet and an unfed control were run.

A pre-trial was undertaken to set an experiment feed rate. Duplicate buckets containing experimental oysters (1 mL) were fed at a range of *Thalassiosira pseudonana* concentrations for two days. Algal cell concentration immediately after addition and after 24 h feeding by the oysters was used to measure consumption rate then related to algal DW. Feed rates (DW) were set at an average daily consumption rate of between 80-100%. For live algal diets, cultures were maintained in logarithmic to late-logarithmic growth by semi-continuously harvesting cultures and by subculturing to supply new cultures. DW of cultures were measured daily (section 2.1 i.) to assess the volume of culture required to feed the oysters. The DW of algal concentrates was constant and measured at the start of their use as feed. Each day the total requirement

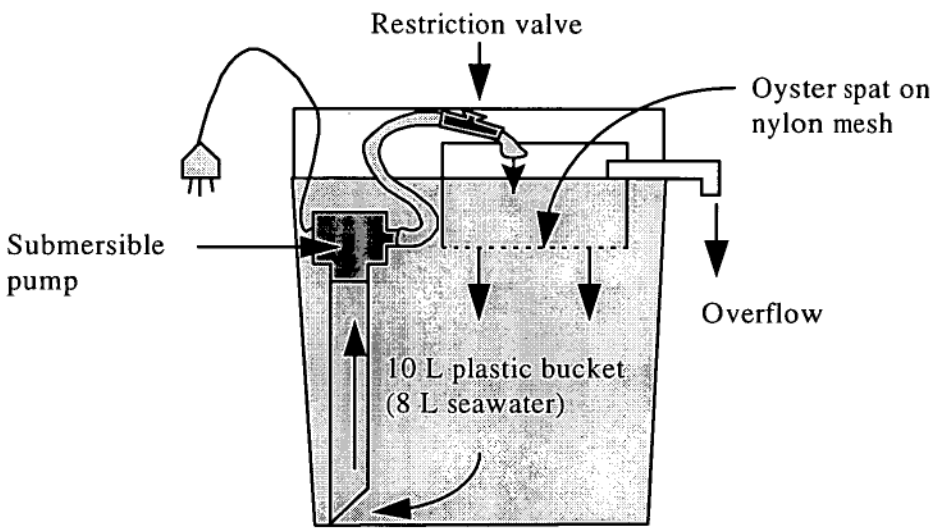
of algal concentrate was resuspended in 30 mL of seawater by gently shaking by hand (30 s) then diluted in enough seawater so that each buckets feed was fed ~100 mL.

At the start of each experiment, five 1 mL samples of oysters were collected as 'day 0' samples. These were rinsed (3 x 10 mL) in Milli-Q water and a subsample of 50 oysters were counted from each sample. The initial samples and subsamples were then dried at 100 °C for 48 h, then weighed to give a DW. The samples were then heated in a muffle furnace (450 °C; 24 h), and re-weighed to determine AFDW. The weight of the subsample was used to estimate the total number of oysters and the individual oyster weight. At completion of the trial oysters from each chamber were collected, rinsed (3 x 10 mL) in Milli-Q water and a representative subsample of 50 oysters were removed for biochemical analysis. The remainder were dried and ashed to determine DW and AFDW. Oyster growth rates were determined from measurements of the initial and final DW and AFDW to give an instantaneous growth rate (k) in d^{-1}.

$$k = (\ln W_f - \ln W_i) / t$$

where, W_f and W_i refer to final and initial oyster weights and t = duration of the experiment in days.

A)



Downweller, recirculating operation

B)

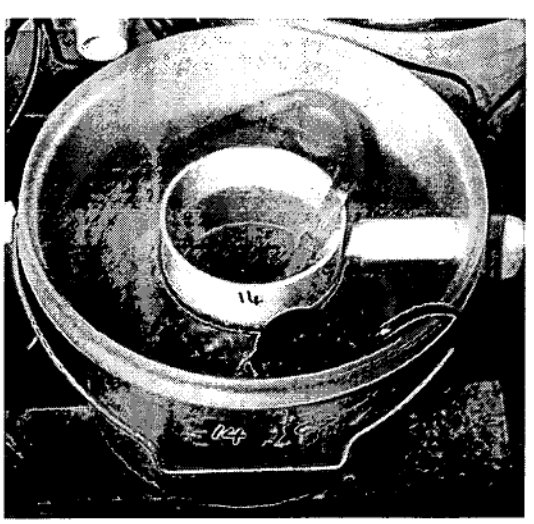


Figure 2.1 **A)** Cross-sectional view of an experimental oyster cultivation unit, operating as a recirculating downweller.
B) An operational downweller unit with juvenile oysters on the mesh screen.

2.1 iii. Bacteriology

Bacteriological media was used to check the axenic status of algal cultures, to indicate the effectiveness of filtration (0.2 μm) of seawater, to check for the presence of potentially harmful *Vibrio* sp. and to quantify the number of viable bacteria in algal cultures and concentrates. It was not used to identify bacterial species other than the partial identification of *Vibrio* sp.

For non-selective growth of bacteria, Difco Marine Agar 2216 with vitamins (MAV) or seawater agar with vitamins (SWAV) was used. MAV was reconstituted according to product directions and additional agar (Oxoid No 1) was added if a stronger gel was required. SWAV was made in filtered seawater by addition (1 $g \cdot L^{-1}$) of bacteriological peptone (Oxoid) and yeast extract (Oxoid) and solidified by agar (Oxoid No 1). Vitamins at f/2 concentration (Table 2.1, stock 6), were added to MAV and SWAV. Thiosulfate-citrate-bile salt-sucrose Cholera medium (TCBS, Oxoid) was used as a selective media for *Vibrio* sp. It was dissolved (50:50, filtered seawater:deionised water), according to directions.

For alga culture volumes > 20 L, autoclave sterilisation of seawater is not practical and filtration was used to reduce background bacterial numbers. Raw seawater was pumped through a series of filters of decreasing pore size:

1	10	μm nominal pore size
2	1	μm nominal pore size
3	1.2	μm absolute pore size
4	0.6	μm absolute pore size
5	0.45	μm absolute pore size
6	0.20	μm autoclaved filter, housing and transfer lines.

A sample (500 mL) of filtered water was collected in a sterile bottle and duplicate aliquots (100 mL) filtered through a sterile 47 mm filter-housing fitted onto a 0.2 μm filter (Lewis *et al.*, 1986). Filters were placed on the surface of a MAV or SWAV plate and incubated (20 ± 2°C) for 5-7 days. Colonies were then counted and expressed as number of bacteria per 100 mL.

During isolation and axenisation, algal isolates were grown on solid medium before being transferred to liquid media and maintained. Checks for bacterial contamination were conducted during axenisation and at each routine subculture of the liquid stock cultures. Colonies were picked on solid media algal using a platinum loop and streaked in a 'zigzag' pattern on to MAV or SWAV. When stock cultures of algae were subcultured, 1-2 drops were aseptically transferred to a MAV or SWAV plate and spread using an L-shaped glass rod. All bacterial plates were incubated (20 ± 2°C) for 5-7 days to allow time for growth of any bacteria. These were primarily positive/negative tests but a qualitative assessment of contamination is possible by noting the variety and density of bacterial growth.

Bacteria in algal cultures and their concentration and growth in algal concentrates was measured as viable bacteria growing on MAV or SWAV plates. A sample (1 mL) of algal culture or liquid concentrate was serially diluted in dilution-seawater (Peptone, 1 $g \cdot L^{-1}$; polyoxyethylene sorbitan mono-oleate (Tween 80), 5 $mL \cdot L^{-1}$) to 10^{-6} for culture samples and 10^{-8} for liquid cell concentrates. An aliquot (100 µL) from each dilution, including the undiluted culture (10^{0}), was spread-plated on to individual MAV or SWAV plates. Aliquots from 10^{0} - 10^{-3} dilutions were also spread-plated on to TCBS media to determine the presence and relative concentration of *Vibrio* sp. All plates were incubated at 20 ± 2°C. TCBS plates were counted after 2-3 days and MAV or SWAV plates counted after 5-7 days. Plates containing 20-200 colonies were used to calculate the original bacterial concentration:

eg. · 100 µL of dilution 10^{-3} → 56 colonies

→ 56 x 10 = 560 colonies·mL^{-1} in 10^{-3} dilution

→ 560 ÷ 10^{-3} = 5.6 x 10^{5} viable bacteria·mL^{-1} of culture

For algal pastes, an aliquot (~ 0.1-0.2 g wet weight) was aseptically added to a pre-weighed sterile, capped, 50 mL plastic tube. The tube contained sterile glass beads (~ 0.5 g, 0.5 mm diameter) to aid the rapid resuspension of the paste. The tube was re-weighed to accurately measure the weight of the sample then the contents of a MacCartney bottle (9 mL) of dilution-seawater was added. The tube was mixed by

shaking, until no clumps were visible when held up to strong light. This was then serially diluted to 10^{-8} dilution and immediately plated out as for culture and liquid concentrate samples. Bacteria numbers were expressed as viable bacteria per gram of algal paste.

2.2. Biochemical Analysis

2.2 i. Chlorophyll *a*

Duplicate algal culture samples (10-100 mL) were filtered onto glass fibre filter papers (Whatman, GF/C) and extracted with 100% acetone (2.7 mL for 2.5 cm filters, 4.5 mL for 4.7 cm filters). Extraction was facilitated with sonication (30 sec; 50-100W) using a Braun Labsonic 1510 fitted with a 4 mm diameter probe. The acetone was adjusted to 90% by addition of 0.2 mL Milli-Q water (assuming 0.1 mL and 0.3 mL of water on 2.5 cm and 4.7 cm filters). Algal concentrates, of liquid concentrate (1 mL) or paste (0.10-0.20 g), were dispensed into screw-capped plastic tubes and extracted with 10 mL of 100% acetone. After sonication, extracts were adjusted to 90% acetone after accounting for dilution by the quantity of material extracted. Some chlorophytes and eustigmatophytes were poorly extracted with acetone due to their resistant cell walls. Cultures or concentrates of these algae were extracted in 5-10 mL methanol (100%) and then diluted in acetone. Extracts were left on ice in the dark for 30 min then centrifuged (670g, 5 min) and, if required, diluted with 90% acetone. Chlorophyll *a* in the supernatant was determined spectrophotometrically using the appropriate equations of Jeffrey and Humphrey (1975):

Chlorophytes, prasinophytes, eustigmatophytes	Chl *a* = 11.93 A 664 - 1.93 A 647
Diatoms and chrysomonads	Chl *a* = 11.47 A 664 - 0.40 A 630

Selected samples were then scanned (350 - 700 nm) to detect shifts in absorbance spectra associated with chlorophyll degradation products.

For identification of pigments, selected samples were extracted in the dark and analysed using HPLC. Aliquots of culture (50 mL) were centrifuged (670g, 5 min, -10°C) and

the pellet extracted in methanol with sonication. Extracts were then diluted (1:10) in acetone, centrifuged and the supernatant filtered (0.45 μm). Extracts were analysed using a Waters high performance liquid chromatograph comprising a 600 controller, 717 plus refrigerated autosampler and a 996 photo-diode array detector. Pigments were separated using a stainless steel 25 cm x 4.6 mm I.D. column packed with ODS2 of 5 μm particle size with gradient elution as described by Wright *el al.* (1991). The separated pigments were detected at 436 nm and identified against standard spectra using Waters Millennium software.

2.2 ii. Ascorbic Acid

The basis of this assay is the enzymatic oxidation of ascorbic acid to dehydroascorbic acid and subsequent condensation with o-phenylenediamine (OPD) to form the fluorescent derivative, 3-(1,2-dihydroxyethyl) furo[3,4-*b*]-quinoxaline-1-one (Speek *et al.*, 1984). This product is then quantified by HPLC.

An aliquot of culture (50-100 mL) was filtered under vacuum through a glass-fibre filter and placed in a 10 mL plastic centrifuge tube. For algal concentrates, 50-200 mg of a well-mixed sample was weighed directly into the centrifuge tube. Samples were then extracted and derivitised under subdued light at 4°C. To extract samples, 4.0 mL of 3% metaphosphoric acid + 8% acetic acid (MPA), 1.0 mL of water and 20 μL of EGTA-glutathione solution (0.24 M ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N',-tetraacetic acid and 0.20 M glutathione: pH 6.5) was added to each tube. Samples were vortexed (30 s), sonicated (30 s) and then left for 5 min before the mixing steps were repeated and the samples then left for 30 min to complete ascorbic acid extraction.

After extraction, tubes were centrifuged at 2000g for 10 min. To ensure that ascorbic acid was in the quantifiable range for the assay, two aliquots were taken from each supernatant (1.5 and 0.3 mL) and transferred to separate tubes. All volumes were made up to 1.5 mL with the MPA solution and sodium acetate buffer (4.5 M, pH 6.2; 0.4 mL) was added to each tube. All tubes were then equilibrated at 37°C for 10 min prior to incubation with ascorbic acid oxidase. An ascorbic acid oxidase spatula (Boehringer) was added to each tube. Tubes were then incubated for 5 min at 37°C in a shaking water

bath and vortexed once at 2-3 min. The spatulas were removed and 0.25 mL of a freshly prepared solution of 0.1% (w/v) OPD was added. Tubes were incubated for 30 min, then filtered (0.45 μm syringe filter; Millipore) and analysed by HPLC.

A set of standards were generated from an ascorbic acid solution (7 $\mu g \cdot mL^{-1}$) by dispensing duplicate samples of 0, 0.25, 0.50, 0.75 and 1.0 mL into 10 mL tubes. All volumes were made up to 1.0 mL with water and 4.0 mL of 3% MPA and 1.2 mL of sodium acetate buffer added. Each standards was then incubated with an ascorbic acid-oxidase spatula then derivitised with OPD solution (0.5 mL).

A Varian Model 5000 LC HPLC with a C18 Novapak (Waters; 3.9 x 150 mm) column was used to analyse samples. Injected samples (50 μL) were eluted isocratically with 80% of 0.08 M potassium dihydrogen phosphate: 20% methanol (pH 7.8) at a flow rate of 0.8 $mL \cdot min^{-1}$. A Varian Fluorichrom set at an excitation maximum of 355 nm and an emission maximum of 425 nm was used to detect the ascorbic acid derivative peak that was quantified with Varian-Star software.

2.2 iii. Gross Composition (Protein, Lipid and Carbohydrate)

Individual filtered samples of algae were analysed for protein, lipid and carbohydrate according to the following methods. However, daily samples of algae used as oyster diets in feeding experiments were combined into grouped samples that represented specific feeding periods. These combined algal samples and also subsamples of oysters (50-100) taken during and at completion of feeding experiments were firstly extracted using a method adapted from Kochert, (1978) and Whyte, (1987) to form four fractions (protein, lipid, polysaccharide and mono- and oligosaccharides) which were then analysed individually. Filtered algal samples were placed in 10 mL Maxi-vials (Alltech Associates); frozen, wet oysters were ground with a mortar and pestle and transferred to Maxi-vials. Samples were then extracted for lipid (section 2.2 iiib.). The methanol-water phase, left after collection of the chloroform-lipid phase, contained mono- and oligosaccharides and was concentrated under vacuum at 60°C to 10 mL and analysed for carbohydrate (section 2.2 iiic.) The lipid-free residue left in the Maxi-vial was solubilised by repeated (3 x 5 mL) 10 min extractions at 100°C with 1M NaOH.

Combined extracts were diluted to 25 mL with distilled water and subsamples analysed directly for carbohydrate (polysaccharide fraction) and after adjustment of pH to 8-9 for protein (section 2.2 iiia.).

2.2 iiia. Protein

Protein was determined by a modified Lowry *et al.*, (1951) method (Clayton *et al.*, 1988) after initial fractionation of the samples to remove interfering free amino acids. Filtered samples were first extracted in a Potter-Elvenhjem tissue-homogenising tube with 4.4 mL 6% trichloroacetic acid (TCA). After homogenisation, 0.2 mL of sodium deoxycholate was added to assist protein precipitation. Extracts were centrifuged and the supernatant, containing free amino acids, discarded. The protein pellet was resuspended in 4 mL of Milli-Q water and analysed for protein. The pH-adjusted protein fraction from fractionated algal and oyster samples was analysed for protein without any further treatment. Bovine serum albumin (BSA, sigma chemicals A-7030) was used to create a standard curve by adding 100 - 200 μg to 25 mm glass fibre filter papers and extracting as for algal samples. After colour development, samples were measured spectrophotometrically (750 nm) and protein content determined graphically from the BSA standards.

2.2 iiib. Lipid and Fatty Acids

Lipid was determined gravimetrically and occasionally colorimetrically (Kochert, 1978). Filtered algae were placed in 10 mL Maxi-vials (Alltech Associates) and 8 mL $CHCl_3$:MeOH (2:1) added. Tubes were vigorously mixed and allowed to stand for 1 h then centrifuged and the solvent transferred to a 50 mL separating funnel. The filter was re-extracted with a further 5 mL and 4 mL $CHCl_3$:MeOH (2:1) followed by two 4 mL $CHCl_3$:MeOH:H_2O (2:4:1) extractions. All solvent extracts were combined in the separating funnel and the solvent ratio adjusted to $CHCl_3$:MeOH:H_2O (1:1:0.9) The mixture was shaken and then allowed to separate into chloroform and aqueous phases.

The lipid-rich chloroform phase was collected and the aqueous phase rinsed with 2 mL chloroform. The combined chloroform was then evaporated under vacuum (Rotavapour) at 60°C, the lipid residue re-dissolved in 5 mL $CHCl_3$:MeOH (1:1) and again

evaporated. The lipid was then dissolved in chloroform (1-1.5 mL) and transferred to a pre-weighed glass vial and made up to 2 mL with chloroform. A sub-sample was taken for a colorimetric assay of lipid and the remainder evaporated to dryness at 60°C under a stream of nitrogen. To remove residual water the lipid was re-dissolved in approximately 0.5 mL of dichloromethane (CH_2Cl_2) then MeOH and dried under nitrogen at each stage. The vial of lipid was dried to a constant weight at 60°C under vacuum. The final lipid weight was expressed as a percentage of the AFDW.

In samples from early logarithmic phase cultures, the total extracted lipid fraction weighed less than 1 mg. To confirm the accuracy of these low weights, samples were analysed by a colorimetric assay involving the reduction of potassium dichromate (Kochert, 1978). The sub-sample of the lipid taken from the gravimetric method was placed in a 2 mL Mini-vial (Alltech Associates) and evaporated to dryness. A palmitic acid solution (1 $mg \cdot mL^{-1}$) in chloroform was used as a standard. To the dried sample and standards, 2 mL of dichromate solution (2.5 g $K_2Cr_2O_7 \cdot L^{-1}$ H_2SO_4) was added and tubes sealed with a Telfer-lined cap. Tubes were placed in a boiling-water bath for 45 min and mixed two or three times. After cooling, 1.0 mL was removed from each tube and diluted to 10.0 mL with water. The absorbance was then read at 350 nm against a water blank and the unknown lipid determined graphically from the standard curve. Since the assay is based on the reduction of dichromate by lipid, colour intensity is maximal when lipid is minimal.

Fatty acid samples were extracted overnight from algal samples with chloroform-methanol-water (1:2:0.8, v/v/v) (Bligh and Dyer, 1959). The solvent ratio was adjusted to 1:1:0.9 for phase separation and lipids collected in the chloroform phase. The lipids were saponified and the liberated fatty acids were acidified, then esterified (Volkman *et al.*, 1989). After transesterification of the fatty acids to methyl esters they were analysed using a Varian 3410 gas chromatograph equipped with an FID and autosampler injector. The column was a polar 70% cyanopropyl siloxane (BP-X70) fused-silica capillary column (50 m x 0.25 mm I.D.). Samples were injected at 45°C and after 1 min the oven temperature was raised to 120°C at $30°C \cdot min^{-1}$. The final

temperature was maintained for 20 min. Hydrogen was used as the carrier gas. The detector temperature was 260°C. Peak areas were quantified with DAPA software.

2.2 iiic. Carbohydrate

Filtered algae samples were hydrolysed in capped 10 mL polypropylene tubes containing 3.9 mL of 0.5 M H_2SO_4. This gave a total volume of 4.0 mL, as previous assays established wet filters to contain approximately 0.1 mL of water. Tubes were incubated at 100°C for 4 hr with hourly mixing to facilitate polysaccharide hydrolysis. Samples were then cooled, centrifuged (1500g, 10 min) and a sub-sample analysed by the phenol-sulfuric acid method (Dubois et al., 1956) using a procedure adapted from Kochert, (1978). After colour development samples were measured spectrophotometrically (450 nm) and carbohydrate determined from a glucose standard curve.

For some stationary phase diatom cultures, there appeared to be a significant over-estimation of carbohydrate content. This interference was not apparent in logarithmic phase samples. To reduce interference, samples were extracted to produce a lipid free polysaccharide fraction and a mono-, oligosaccharide fraction. Filtered samples were extracted with 8 mL $CHCl_3$:MeOH (2:1) then 5 mL $CHCl_3$:MeOH:H_2O (2:4:1). The combined solvent extracts were adjusted to a final ratio $CHCl_3$:MeOH:H_2O (1:1:0.9). After phase separation the aqueous phase was collected and assayed directly for carbohydrate. The filter paper containing the polysaccharide fraction was hydrolysed and analysed for carbohydrate.

2.3. Microscopy

2.3 i. Light Microscopy

Cell counts and observations on general condition of algal cultures were done using an Olympus (Vanox) microscope. An inverted microscope (Nikkon- Diaphot) was used for micromanipulation of algal cells during isolation and for examination of algal colonies growing on solid media. Purified isolates were photographed using a Zeiss (Axioplan) microscope with phase and Normaski differential interference contrast (DIC Normaski). The viability of juvenile oysters was determined by examining ~100 spat using a dissection microscope and counting empty shells.

2.3 ii. Scanning Electron Microscopy

For identification of some isolates, logarithmically growing algal cultures were prepared for viewing by scanning electron microscope. A 10 mL sample of culture was centrifuged (170g, 5 min) to provide a 1 mL concentrate. One to two drops of 2 % osmium tetroxide (OsO_4) was added and samples incubated at 4°C for 1 h. The OsO_4 fixes cells by crosslinking unsaturated lipids and proteins by reacting with their double bonds. In doing so the tissue is strengthened so that it can withstand subsequent stages. After Treatment with OsO_4 the samples were dehydrated by rinsing in a series of acetone washes (Table 2.2). Dehydrated specimens were filtered (0.2 µm) and mounted onto SEM stubs, taking care to avoid letting the sample dry. As samples dry the liquid interface moves through the specimen and the surface tension can deform or collapse the cell. Critical point drying minimises damage to the specimen by avoiding surface tension forces. At the critical point of CO_2 (about 32°C and 1100 psi) the density of liquid and vapour are the same and liquid CO_2 instantly become a gas of equal density, avoiding the formation of an interface. Liquid CO_2 is not miscible with water so specimens must be dehydrated before drying. Dehydrated specimens in acetone were placed in the critical-point drying apparatus (Blazers CPD 030) and the chamber filled with liquid CO_2. The acetone was removed by repeatedly flushing the chamber with liquid CO_2, taking care not to allow the liquid CO_2 level to fall below the specimen and damage the cell structure. After removal of the acetone the chamber was filled with liquid CO_2 and sealed. The chamber was then slowly heated through the critical point.

Once past the critical point the CO_2 vaporised, leaving the samples dry. The gas was then slowly bled off taking care to avoid condensing the CO_2. The samples were then removed and sputter coated.

Table 2.2 Sample dehydration sequence for SEM samples.

	Treatment	Time	Temp.
Fix	1-2 drops 2 % OsO_4 per mL of cell suspension	1 hour	4 °C
Rinse 1	Filtered seawater	5-10 min	R.T.
Rinse 2	Filtered seawater : Milli-Q H_2O (50:50)	5-10 min	R.T.
Rinse 3	Milli-Q H_2O	5-10 min	R.T.
Rinse 4	Milli-Q H_2O	5-10 min	R.T.
Dehydrate	30 % Acetone	5-10 min	R.T.
	50 % Acetone	5-10 min	R.T.
	70 % Acetone	5-10 min	R.T.
	100 % Acetone	10 min	R.T.
	100 % Acetone	Proceed to next step	

R.T. = room temperature

Sputter coating applies a conductive layer to the surface of the specimens. The coating (approximately 20 nm) is required to prevent a build-up of negative charge while the electron beam is scanning the specimen. The specimens were set in the anode and the sputtering metal (Platinum) forms the cathode. The chamber was put under low vacuum in the presence of Argon and a charge applied causing a discharge of platinum that coats the sample. Specimen samples were then viewed using a Phillips SEM 515 scanning electron microscope.

2.3 iii. Transmission Electron Microscopy

Some algal isolates were identified to genus level from SEM images. However, examination of the silica frustule was needed for identification to species level. To achieve this, samples were cleaned of organic mater and examined by TEM. To remove organic material, samples were treated with potassium peroxodisulfate ($K_2S_2O_8$) (Ma and Jeffrey, 1978). This is a relatively gentle oxidation procedure, suitable for very small diatoms, which are easily destroyed by other procedures.

In two 10 mL samples of culture, 100 µg and 250 µg of $K_2S_2O_8$ was dissolved, then samples were incubated overnight at 60°C. To confirm oxidation of all organic matter, samples were examined microscopically and a further 100 µg of $K_2S_2O_8$ was added to samples where organic matter was still present. Cleaned specimens were centrifuged (170g, 5 min) and rinsed three times in 10 mL volumes of Milli-Q H_2O to remove salts. The washed frustules were re-suspended in 1.0 mL of Milli-Q H_2O for mounting.

Prepared grids (3.05 mm copper grids, 150 hexagonal meshes/inch) were supplied for mounting the cleaned frustules. These had been coated with 0.75 % Formvar (polyvinyl formal) solution in dichloromethane. A Formvar film was first cast on to a glass slide, dried, then floated on to water where the grids were laid on to the film. The film with grids was removed, dried, then coated with a thin film of evaporated carbon (~ 5 nm). Individual grids were removed from the film by gently scoring around the grid then lifting it off with fine forceps. The grids were then stuck, by their edge, to a strip of sticky tape attached to a microscope slide. A small drop of diatom frustule suspension was placed on the grid, which was left undisturbed for 30 min to allow specimens to settle. Most of the drop was then drawn off using fine dental swabs. The grids, attached to the slide, were covered with a glass Petri dish and allowed to fully dry before viewing. A Phillips CM 100 (80 kV) transmission microscope was used to examine the grids.

2.4. Statistics

Statistical analysis was performed using the statistical program, Minitab release 11. Data (biochemical composition of algae and oysters, oyster growth and feed rates) were examined for equality of variances (residual plots) and data transformed where necessary before further analysis. An analysis of variance (one-way ANOVA) was then performed and data showing significant differences ($P<0.05$) across treatments was analysed by pairwise multiple comparison of means (Fisher's protected least significant difference; PLSD).

Regression analysis was undertaken on data to determine correlations with time, diet or composition. Stepwise regression was used to determine the principal diet component affecting oyster growth. The general linear model (GLM) was used to compare regression lines for significant differences between slopes.

Chapter Three

Isolation and Characterisation of Australian Microalgae

Chapter 3

Isolation and Characterisation of Australian Microalgae

3.1. *Introduction*

Species suitable for aquaculture are selected for their overall nutritional value and also their ease of culture. Traditionally grown species are readily available from commercial and research collections including the CSIRO Collection of Living Microalgae in Hobart, Australia. Many are available as axenic cultures, but new isolates have to undergo a lengthy isolation procedure before being available in this form.

Collection of isolates involves sampling habitats where conditions may be selecting for properties deemed desirable in a species. These samples contain many organisms besides the desired algae and need to be purified. This involves successive plating-out of isolates onto nutrient agar until an axenic colony suitable for inoculation into liquid media is obtained. Several steps can be added to reduce the initial contamination load, which is usually largely bacterial. These can include use of antibiotics, selective filtration or centrifugation and using the phototaxis response of many microalgae. Purified cultures are then maintained as stock cultures on agar slopes, liquid media or occasionally under liquid nitrogen. Purification can be a lengthy process as initial cell numbers are usually low relative to contaminating organisms.

The purpose of studies described in this chapter was to isolate and characterise endemic phytoplankton and to identify potentially useful species for aquaculture. Such species would need to be highly productive with a nutritional profile matched to the nutritional requirement of the consumer species.

3.2. *Materials and Methods*

3.2 i. Isolation and Purification of Microalgae

Phytoplankton were isolated from seawater samples collected from Little Swanport (42° 20': 147° 56'), on the East coast of Tasmania, Australia. The samples formed part of a CSIRO weekly monitoring of the flow-through water at Shellfish Culture's oyster nursery located at the mouth of Little Swanport River (Brown and McCausland, 1998). Isolations were from water samples collected in early autumn (March-April 1995) when average water conditions were: temperature 15°C, salinity 35.40 ppt and total chlorophyll *a* 3.79 $\mu g \cdot L^{-1}$ of which 50% belonged to the < 20 μm fraction. A single isolation was also made from a non-characterised water sample taken from the Derwent River in Hobart, Tasmania, Australia.

Water samples were pre-filtered through 20 μm and 5 μm nylon screens to remove debri and large algae such as chain forming diatoms. They were then gently concentrated using a continuous flow plankton centrifuge (manufactured by CSIRO). After centrifugation of a 2 L sample, and gentle resuspension of the resulting pellet, a 20 mL algal concentrate was obtained and transferred to a Petri dish for micromanipulation. The Petri dish was scanned with an inverted microscope (Nikkon-Diaphot) and selected algae extracted using very fine, extruded glass pipettes. Algae were transferred within micro-drops (~ 1 μL) onto f/2 nutrient (0.8% agar) plates (section 2.1 i.). Up to 40 such drops were placed onto a plate before it was sealed with parafilm and placed in a growth cabinet. The cabinet was set at 15°C with a 12:12 h light:dark cycle and an average light intensity of 100 $\mu mol\ photon \cdot m^{-2} \cdot sec^{-1}$ provided by cool white fluorescent lights.

Inoculated plates were examined microscopically and when colonies were only a few hundred cells in size they were aseptically picked and streaked on to a new f/2 plate. Transferring colonies at this early stage greatly reduced the bacterial load by avoiding confluence of adjacent bacterial colonies. Isolates were then returned to the growth cabinet and allowed to grow for several weeks before being subcultured. Several of the initial isolations contained two algal species and these were picked separately so that

all were mono-algal. Isolates were then maintained by routine 16 streak inoculation onto new f/2 agar plates.

By repeated picking of very young algal colonies of just a few hundred cells, several of the isolates were rendered axenic. However, on many plates, motile bacteria were able to swarm over the moist agar surface and contaminate the algal colonies. To reduce swarming, the agar concentration was increased from 0.8% to 1.4% and 2%. Contaminated algal isolates were streaked on to f/2 agar plates containing the higher agar concentrations. If colonies grew free of bacteria they were picked and plated back onto 0.8% agar plates.

Antibiotics were used to clean-up the remaining isolates (Table 3.1). Stock solutions of antibiotics were made in Milli-Q water and filter-sterilised, except for chloramphenicol which was dissolved in ethanol. They were added to cooled, autoclaved, f/2 agar just prior to pouring into Petri dishes.

Table 3.1 Antibiotic combinations and working concentrations used in agar plates.

Antibiotic Combination			Antibiotic Effect		Concentration
Grouping	Antibiotic	Sigma Chemicals ID #	Action	Site	($mg \cdot L^{-1}$ agar)
1	Penicillin-G (Na-salt)	PEN-NA	Bactericidal	Cell wall	400
	Kanamycin A (SO_4)	K 4000	Bactericidal	Ribosome (70S)	100
	Streptomycin (SO_4)	S 6501	Bactericidal	Ribosome (30S)	200
2	Neomycin (SO_4)	N 1876	Bactericidal	Ribosome	100
	Gentamicin (SO_4)	G 3632	Bactericidal	Ribosome (50S)	100
3	Chloramphenicol	C 0378	Bacteriostatic	Ribosome (50S)	100

For maximum antibiotic toxicity, bacteria need to be undergoing cell division or protein synthesis, which requires a carbon source. Nutrient agar plates (f/2), solidified using high purity agar contain almost no digestible carbon source. Therefore, a filter-sterilised solution of sucrose was added as a carbon source (100 μM). To aid diffusion of the

antibiotics, the agar concentration was reduced from the normal 0.8% to 0.7% for plates containing antibiotic combination 1, and to 0.6% for plates containing combination 2 and chloramphenicol.

Contaminated algal isolates were streaked onto an agar plate containing antibiotic combination 1. To increase the likelihood of making algae axenic, an algae/agar plug and a dense algal inoculation were also applied. The streaked algal inoculum was not transferred between antibiotic plates but was used to isolate single colonies, if tests showed them to be axenic. The dense inoculum and algae/agar plug were transferred to plates containing antibiotic combination 2 and finally to the chloramphenicol plate. They were left in contact with each antibiotic plate for 48 hr. At each transfer stage, a portion of the densely inoculated algae was rinsed in 2 mL of sterile seawater. The resulting suspension was spread-plated onto a SWAV plate, to check for bacterial growth, and an f/2 (0.8% agar) plate to isolate potentially axenic algal colonies. Axenic algal isolates were selected from cells exposed to the minimum combinations of antibiotics that achieved bacteria free colonies. To check axenic status, cultures were examined using phase contrast microscopy (Zeiss axioplan).

To initiate liquid cultures, a small piece of axenic agar/algae was transferred to liquid f/2 media. The pieces of agar were floated on the surface of 2 mL of sterile f/2 media in a 24-well plate to minimise osmotic shock to the algae. The plate was placed in a growth cabinet and after 2-3 days each 2 mL algal culture was aseptically transferred to 75 mL of f/2 media in a 125 mL Erlenmeyer flask. These cultures became the stock cultures and were maintained as outlined in section 2.1 i.

3.2ii. Characterisation and Identification of Isolates

All isolates except *Minidiscus trioculatus* CS-434 were characterised by quantifying their growth rate and biochemical composition in logarithmic and stationary phases of growth. For each isolate, triplicate, 1.5 L cultures in 2 L Erlenmeyer flasks were inoculated (section 2.1 i.). Inoculum cells were pre-adapted by growing an inoculum culture under identical conditions. Growth rate and carrying capacity (cell concentration in stationary phase, cells·mL^{-1}) was determined from cell counts and biochemical composition related to DW and AFDW (section 2.1 i.). A set of samples was taken from

cultures in mid-logarithmic and early stationary phases of growth. Aliquots of culture were filtered onto a set of glass fibre (GF/C) filter papers for biochemical analysis:

- 25 mL (2.5 cm filter) Protein analysis (Section 2.2 iiia.)
- 25 mL (2.5 cm filter) Carbohydrate Analysis (Section 2.2 iiic.)
- 100 mL (4.7 cm filter) Lipid analysis (Section 2.2 iiib.)
- 200 mL (4.7 cm filter) Fatty acid analysis (Section 2.2 iiib.)

Two spare 25 mL samples (2.5 cm filter paper) were also collected. Samples were stored at -20°C except for fatty acid samples which were stored in liquid nitrogen. All samples were analysed within 6 months. Fatty acids were analysed in logarithmic and stationary phase of only the four fastest growing isolates. Isolates were examined using light, SEM, and TEM (section 2.3.), and isolates identified from diatom reference texts (Round *et al.*, 1990; Hasle and Syvertsen, 1997) and personal communications (G.M. Hallegraeff).

3.3. Results

3.3 i. Algal Isolates

A total of 16 microalgae were isolated and made axenic. Theses included 11 diatoms, 2 chlorophytes, 1 prymnesiophyte, 1 cryptophyte and 1 green flagellate.

The prymnesiophyte and green flagellate were axenised and grew readily on f/2 agar plates. However, neither isolate transferred successfully to liquid media and although maintained on agar, they were not analysed further. The cryptophyte was not axenised and was discarded because of its poor growth. The 11 diatoms and 2 chlorophytes were maintained as a collection.

3.3ii. Identification

The pigment profiles of isolates CS-436 and CS-437 were used to classify them to class level. Their pigment profiles were similar with both chlorophyll *a* and *b* present and lutein as the dominant xanthophyll. Small amounts of 9' cis-neoxanthin, violaxanthin and ß, ß-carotene were also present (Fig. 3.1 and 3.2).

The 11 diatom isolates were identified using light microscopy and TEM (Fig. 3.3 to 3.12). Isolates were identified as:

- *Minidiscus trioculatus* (F.J.R. Taylor) Hasle (3 strains)
- *Nitzschia* cf. *paleacea* Grunow (3 strains)
- *Attheya septentrionalis* (Østrup) Crawford
- *Entomoneis* cf. *punctulata* Osada and Kobayashi
- *Extubocellulus spinifera* Hargraves and Guillard
- *Papiliocellulus simplex* Garner and Crawford
- *Thalassiosira oceanica* Hasle

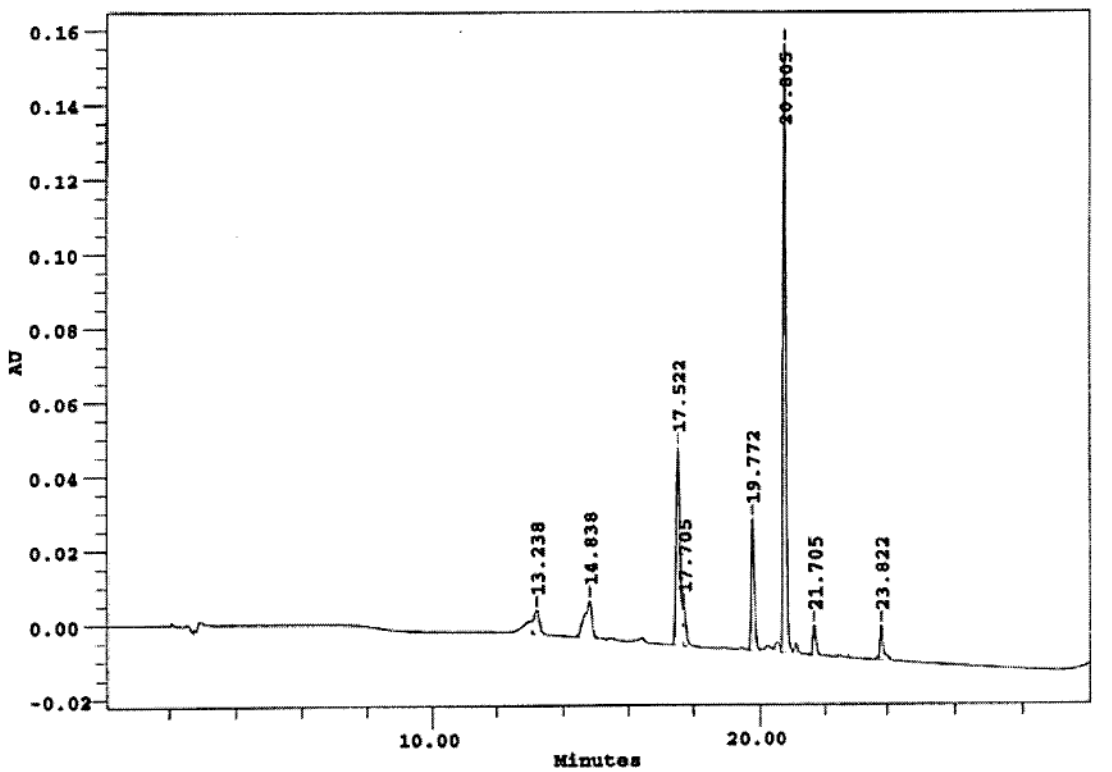


Figure 3.1 HPLC chromatogram of pigment extract of *Stichococcus*-like isolate CS-437. AU (y-axis scale) = absorbance units.

	Retention time (min)	Pigment	Area (μVsec)
1	13.238	9' cis-neoxanthin	77968
2	14.838	Violaxanthin	160763
3	17.522	Lutein	475728
4	17.705	Zeaxanthin	68900
5	19.772	Chlorophyll *b*	229002
6	20.805	Chlorophyll *a*	1015036
7	21.705	(Chlorophyll *a* spectra)	48824
8	23.822	β,β-carotene	70063

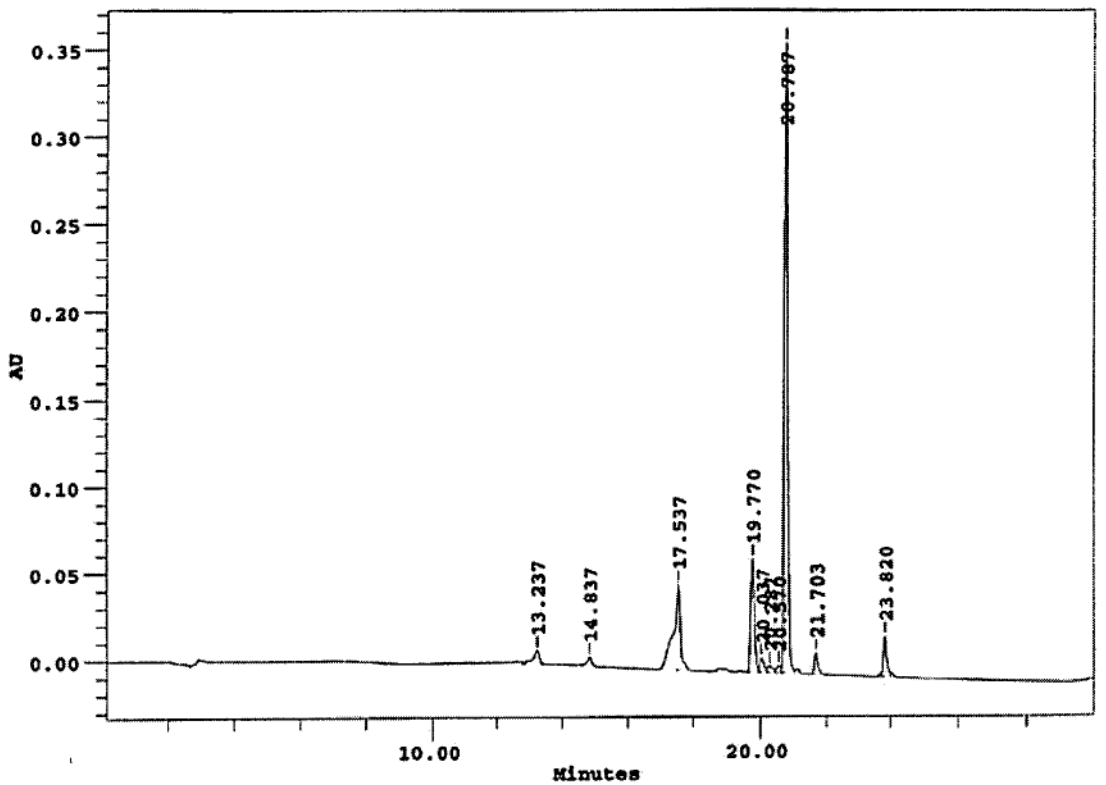


Figure 3.2 HPLC chromatogram of pigment extract of *Chlorella*-like isolate CS-436. AU (y-axis scale) = absorbance units.

	Retention time (min)	Pigment	Area (μVsec)
1	13.237	9' cis-neoxanthin	119745
2	14.837	Violaxanthin	56334
3	17.537	Lutein	777474
4	19.77	Chlorophyll *b*	515633
5	20.037	(Chlorophyll *a* allomer spectra)	76275
6	20.287	Chlorophyll *a* allomer	48235
7	20.57	(Chlorophyll *a* allomer spectra)	35526
8	20.787	Chlorophyll *a*	2401661
9	21.703	(Chlorophyll *a* allomer spectra)	88142
10	23.82	β,β-carotene	157345

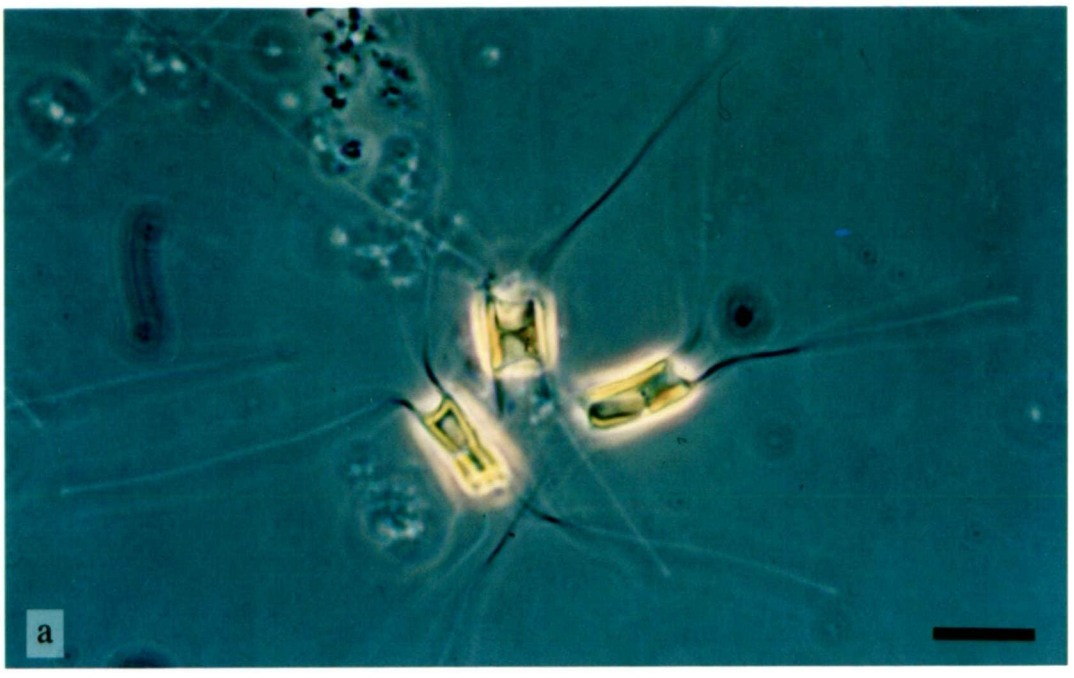
Figure 3.3 Light and transmission electron micrographs of isolate CS-425.

Attheya septentrionalis (Østrup) Crawford (Plate 47).

Basionym: *Chaetoceros septentrionalis* (Østrup).
Synonym: *Gonioceros septentrionalis* (Østrup) Round, Crawford and Mann.

Crawford *et al.*, 1994.

Fig. a (LM). Phase contrast image of live cells. **Fig. b-c (TEM).** Girdle view showing attachment of the characteristic twisted spines which arise at the poles of the valve and project parallel to the valvar plane, tips of spines are open and thickened (Fig. b, arrow). Detailed structure of the spines (Fig. c). Surface of the valve (Fig. d). -Scale bars: 10 µm (Fig. a), 2 µm (Fig. b), 0.5 µm (Fig. c), 1 µm (Fig. d).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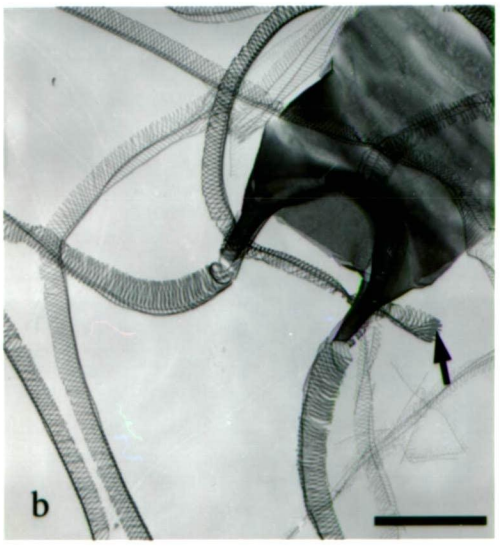
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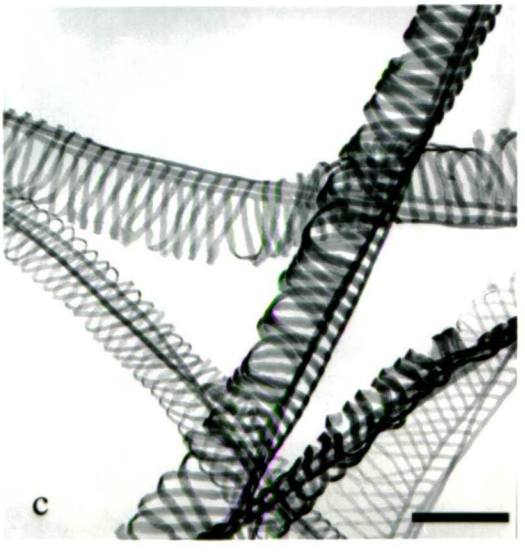
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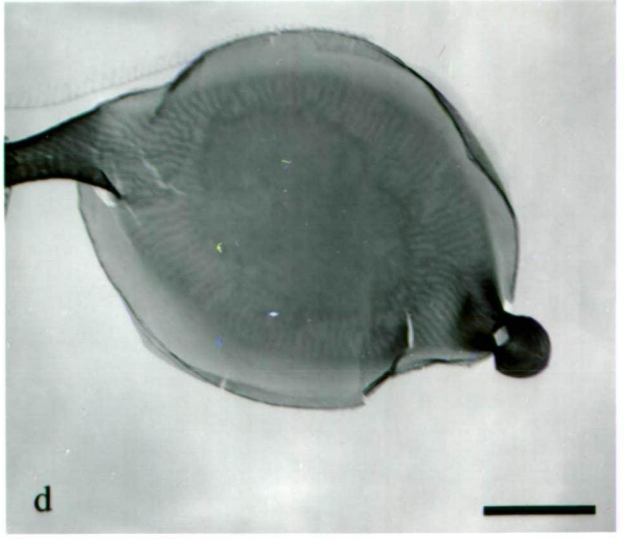
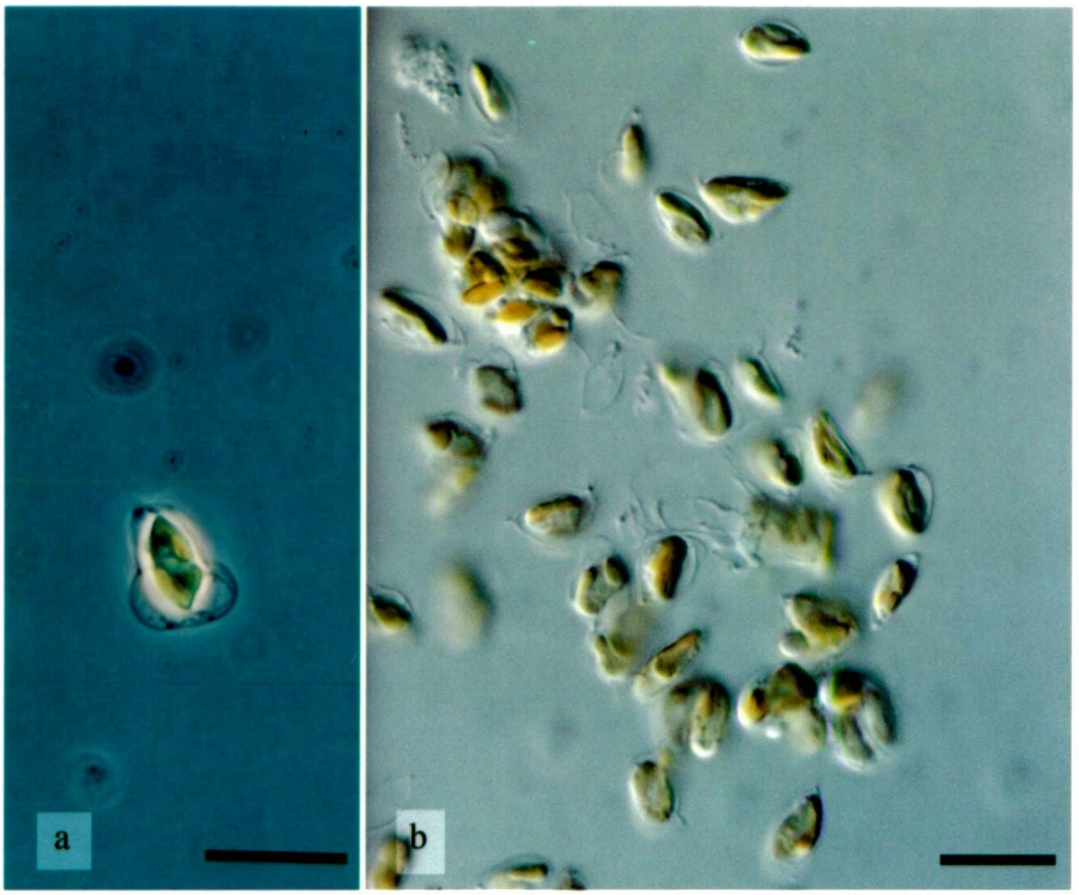

d

Figure 3.4 Light and transmission electron micrographs of isolate CS-426.

Entomoneis cf. *punctulata* Osada and Kobayashi

Osada & Kobayasi 1988. Proc. 10th Diatom Symposium, pp.161-172.

Fig. a & b (LM). Phase contrast image of a solitary cell (Fig. a). DIC (Normaski) image of live cells (Fig. b). **Fig. c & d (TEM).** Pieces of frustule showing the complex structure of the girdle. -Scale bars: 10 μm (Fig. a, b), 2 μm (Fig. c, d).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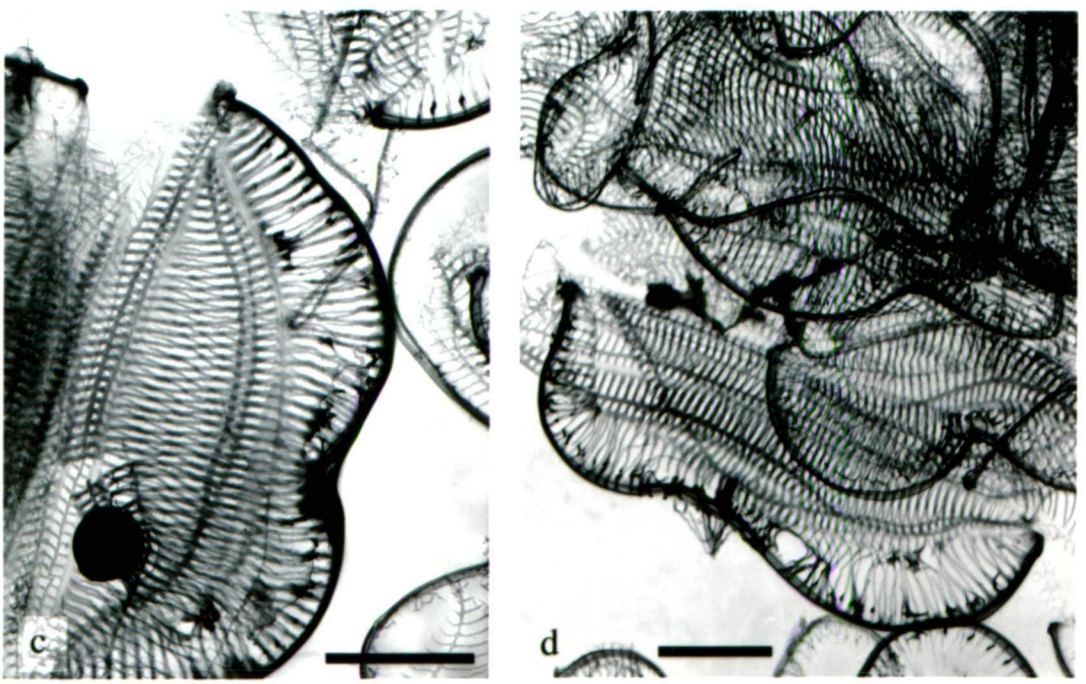
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Figure 3.5 Light and transmission electron micrographs of isolate CS-428.

Extubocellulus spinifera (Hargraves and Guillard)
Hasle, Von Stosch and Syvertsen

Hargraves and Guillard, 1974. Figures 9-12 (LM, TEM); Hasle, Von Stosch and Syvertsen, 1983. Figures 362-390 (LM, TEM, SEM).

Synonym: *Bellerochea spinifera* Hargraves and Guillard

Fig a & b (LM). Single and chains of cells, 5 μm long, 3 μm wide, rectangular in girdle view (Fig. a, phase contrast; Fig. b, DIC -Normaski). **Fig. c & d (TEM).** Valve view of circular cells with a single raised ocelluli with porelli on the valve periphery (arrows). The valve face is perforated by simple areolae with a fine mesh cribrum visible through the areolae (Fig. c). Girdle bands non-porous (Fig. d). -Scale bars: 5 μm (Fig. a, b), 1 μm (Fig. c, d).

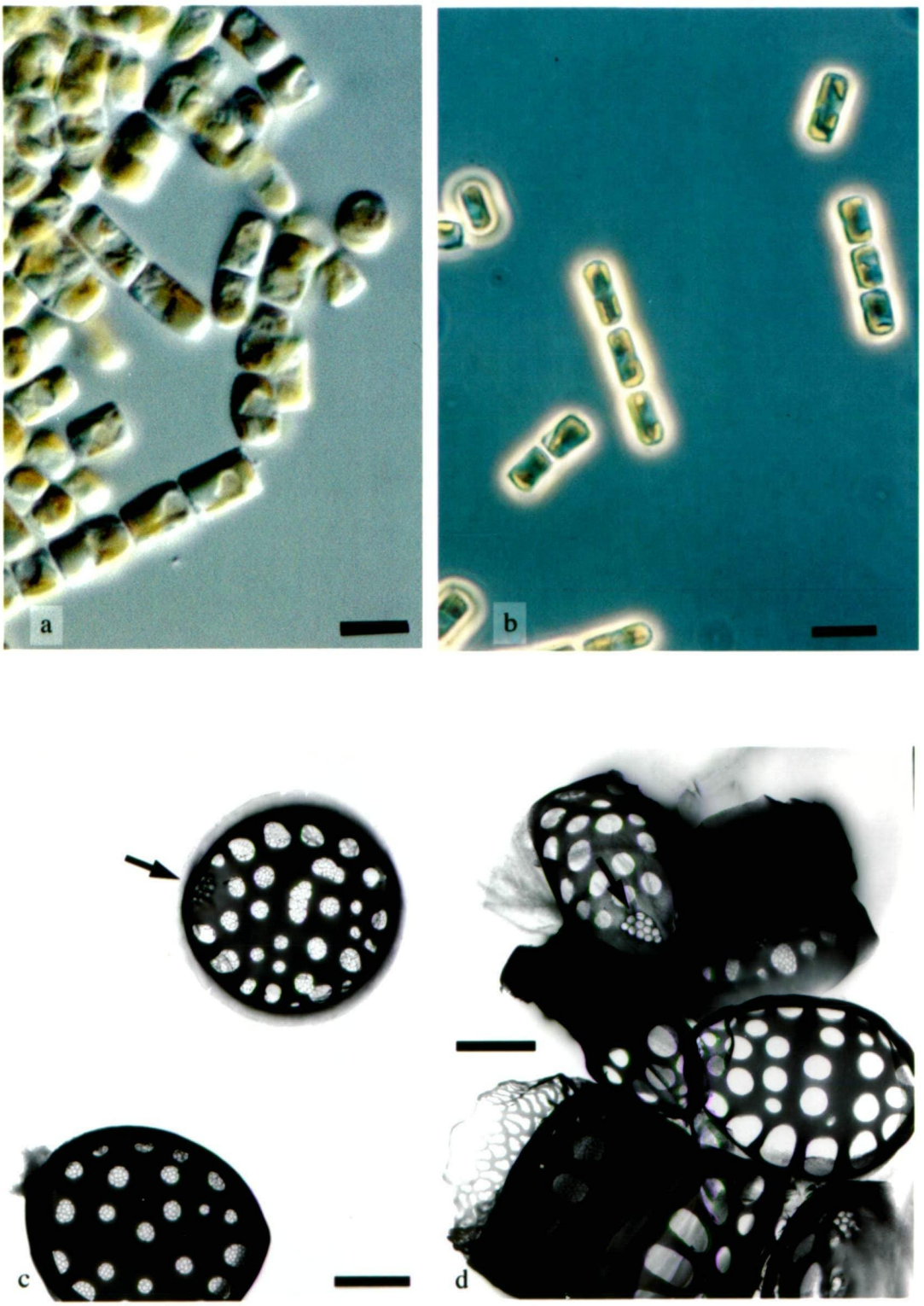
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Figure 3.6 Light and transmission electron micrographs of isolates of CS-432, CS-434 and CS-435.

Minidiscus trioculatus (F.J.R. Taylor) Hasle.

Basionym: *Coscinodiscus trioculatus* F.J.R. Taylor.

Hasle, G.R., 1973.

Fig. a & b (LM). Phase contrast image of CS-435 (Fig. a), appearance is typical of all three isolates. DIC (Normaski) image of CS-434 (Fig. b). **Fig c - e (TEM).** Isolates CS-432 (Fig. c), CS-435 (Fig. d), and CS-434 (Fig. e). Views of valves showing the location of the three processes and prominent haline margin (Fig. c) and the pattern of the areolation (Fig. d). -Scale bars: 5 μm (Fig. a, b), 1 μm (Fig. c), 2 μm (Fig. d, e).

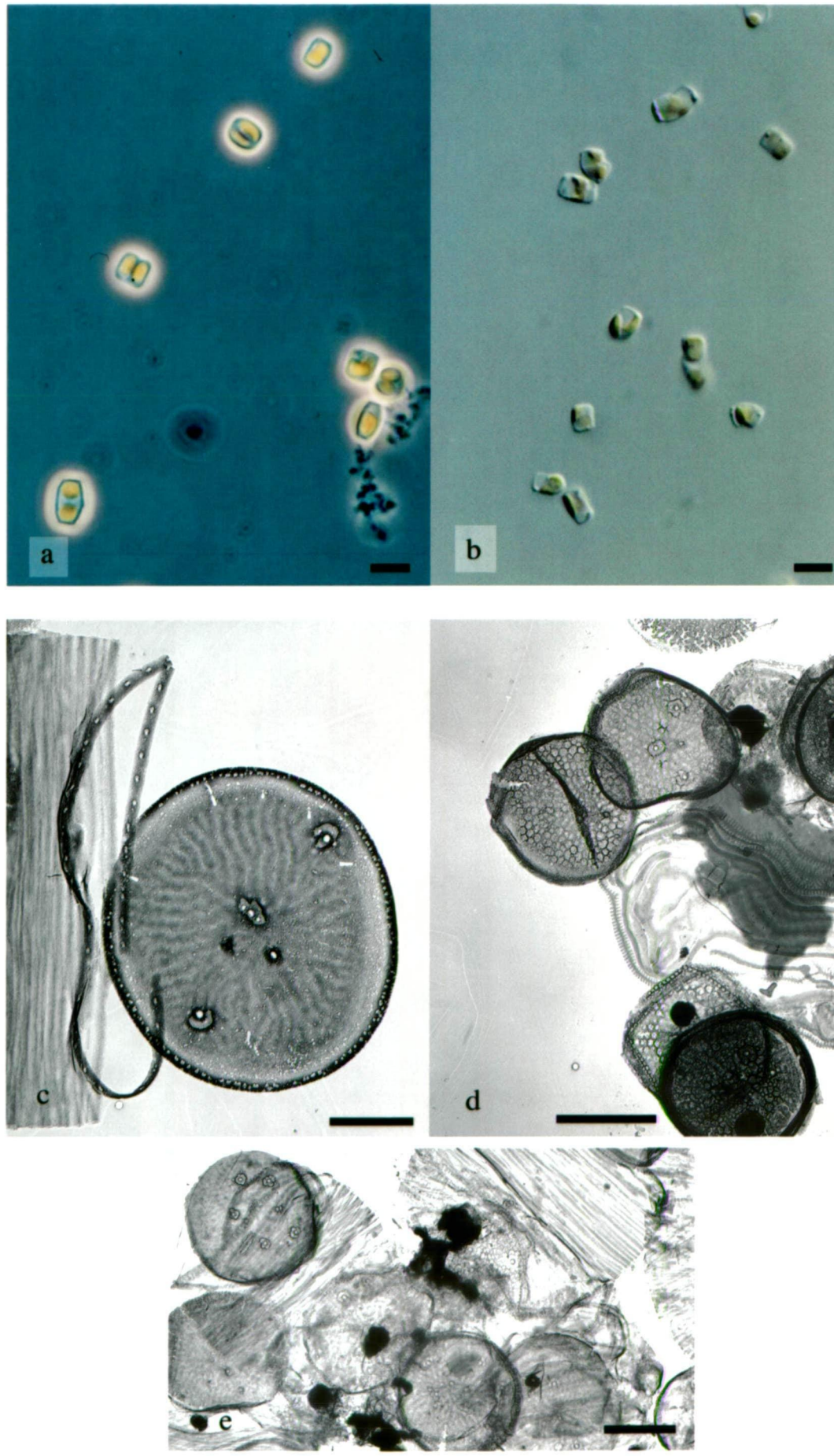
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Figure 3.7 Light and transmission electron micrographs of isolate CS-429.

Nitzschia cf. *paleacea* Grunow

Lange-Bertalot, 1976. Plate 8, Fig. 1-3.

Fig. a (LM) Phase contrast image of live cells showing characteristic pennate diatom shape. **Fig. b & c (TEM)**. Valves showing pattern of striae and areolae. -Scale bars: 10 µm (Fig. a), 2 µm (Fig. b, c).

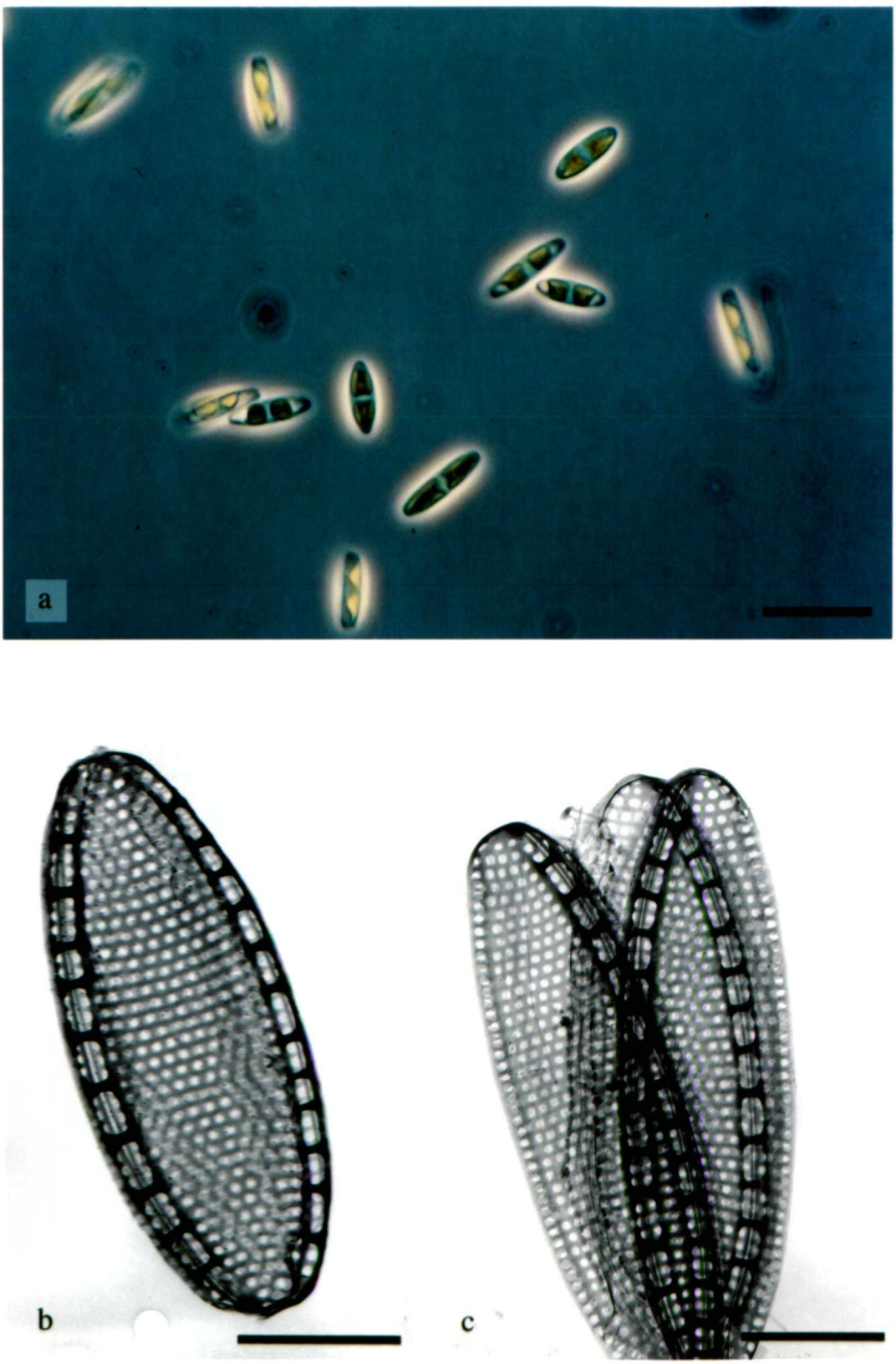
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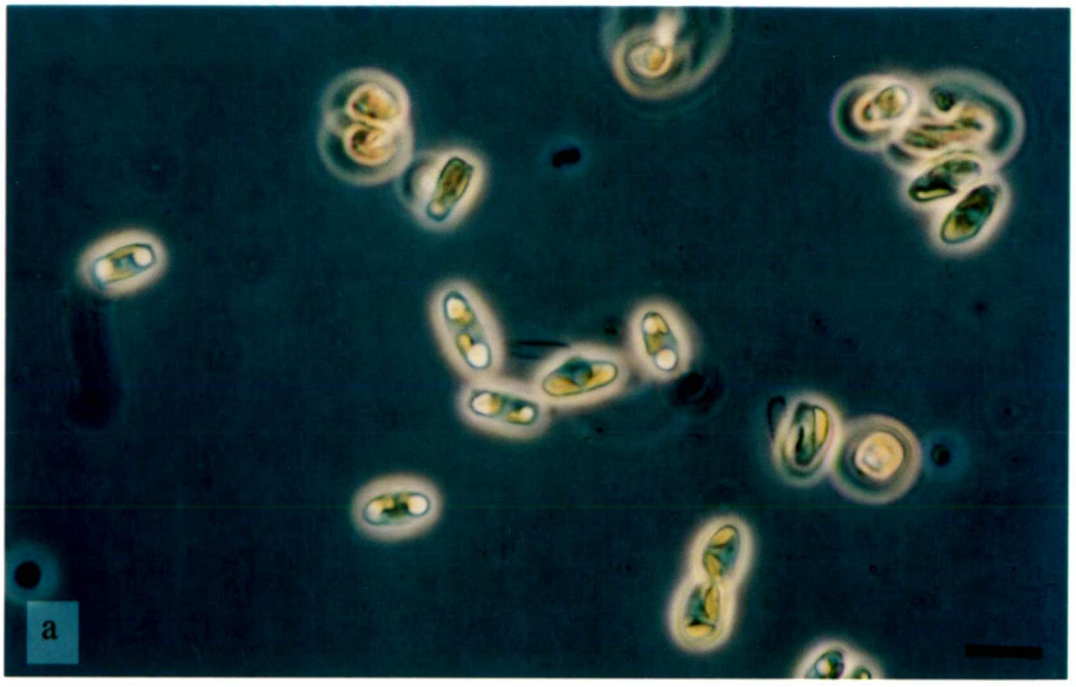
Figure 3.8 Light and transmission electron micrographs of isolate CS-430.

Nitzschia cf. *paleacea* Grunow

Lange-Bertalot, 1976. Plate 8, Fig. 1-3.

Fig. a. (LM) Phase contrast image of live cells showing characteristic pennate diatom shape. **Fig. b-d (TEM)**. Valves showing pattern of striae and areolae. -Scale bars: 6 µm (Fig. a), 2 µm (Fig. b, c), 1 µm (Fig. d).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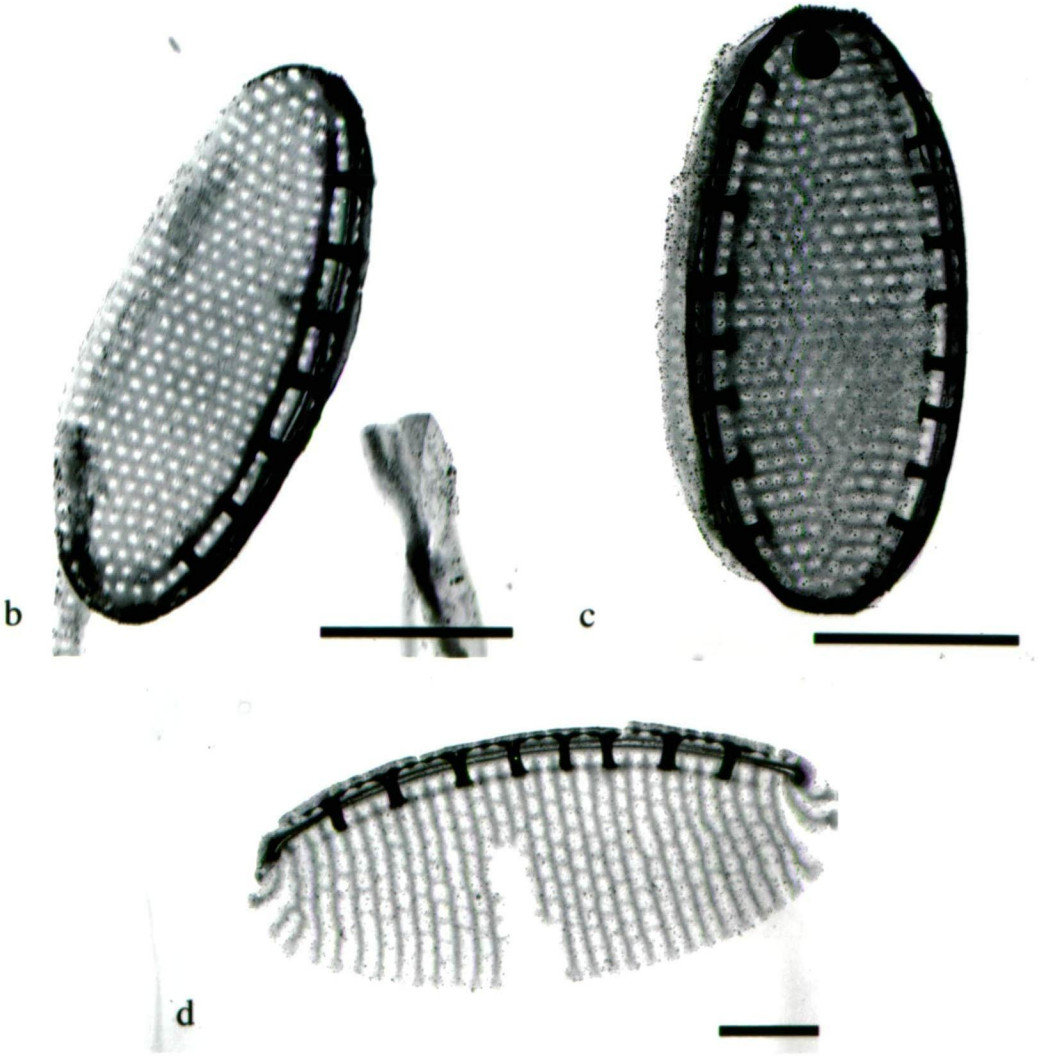
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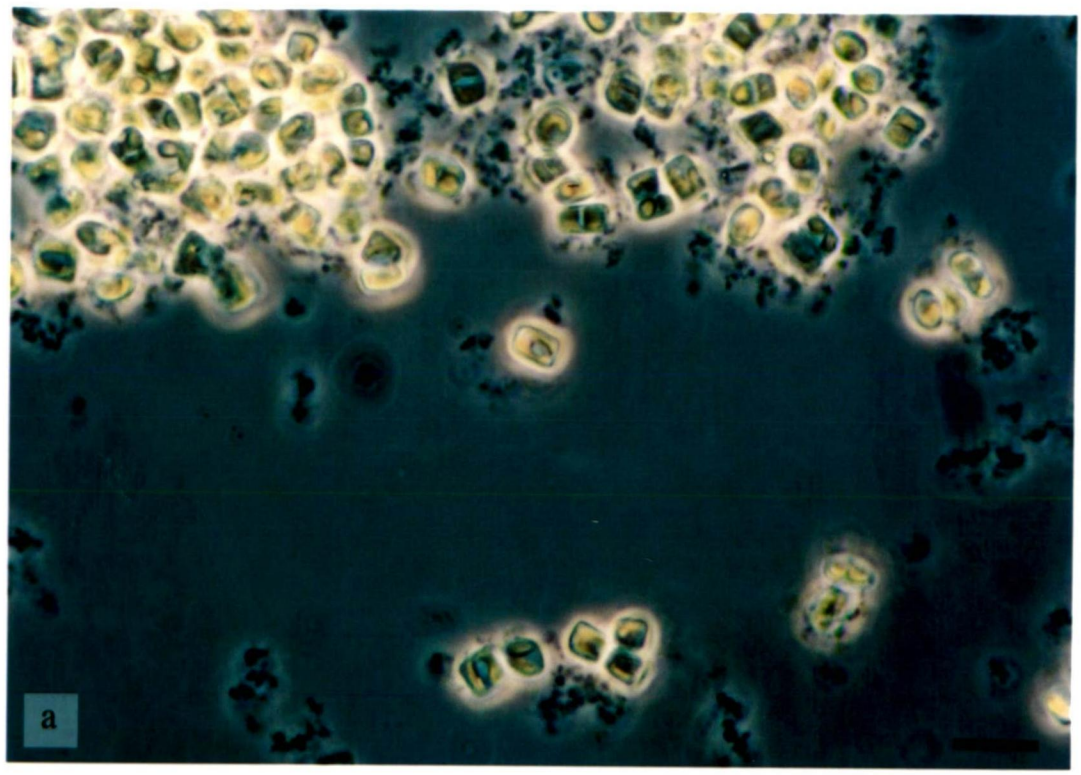
Figure 3.9 Light and transmission electron micrographs of isolate CS-433.

Nitzschia cf. *paleacea* Grunow

Lange-Bertalot, 1976. Plate 8, Fig. 1-3.

Fig. a. (LM) Phase contrast image of live cells showing their strong clumping tendency and uncharacteristic shape. **Fig. b & c (TEM).** Valves showing pattern of striae and areolae and the variation in cell size and shape. -Scale bars: 6 μm (Fig. a), 2 μm (Fig. b, c).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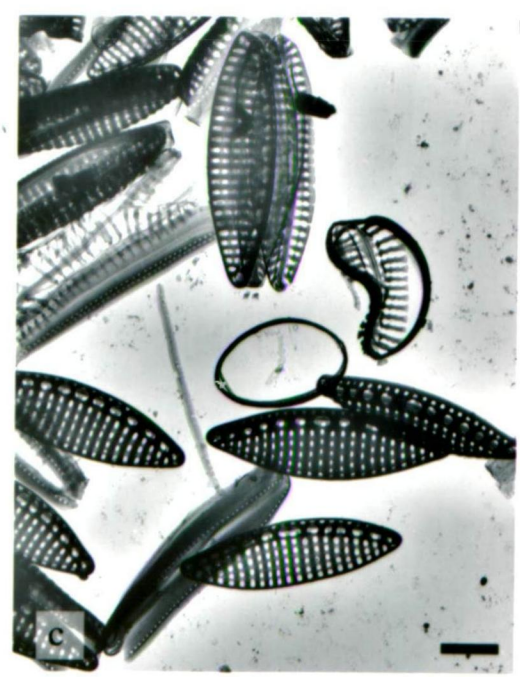
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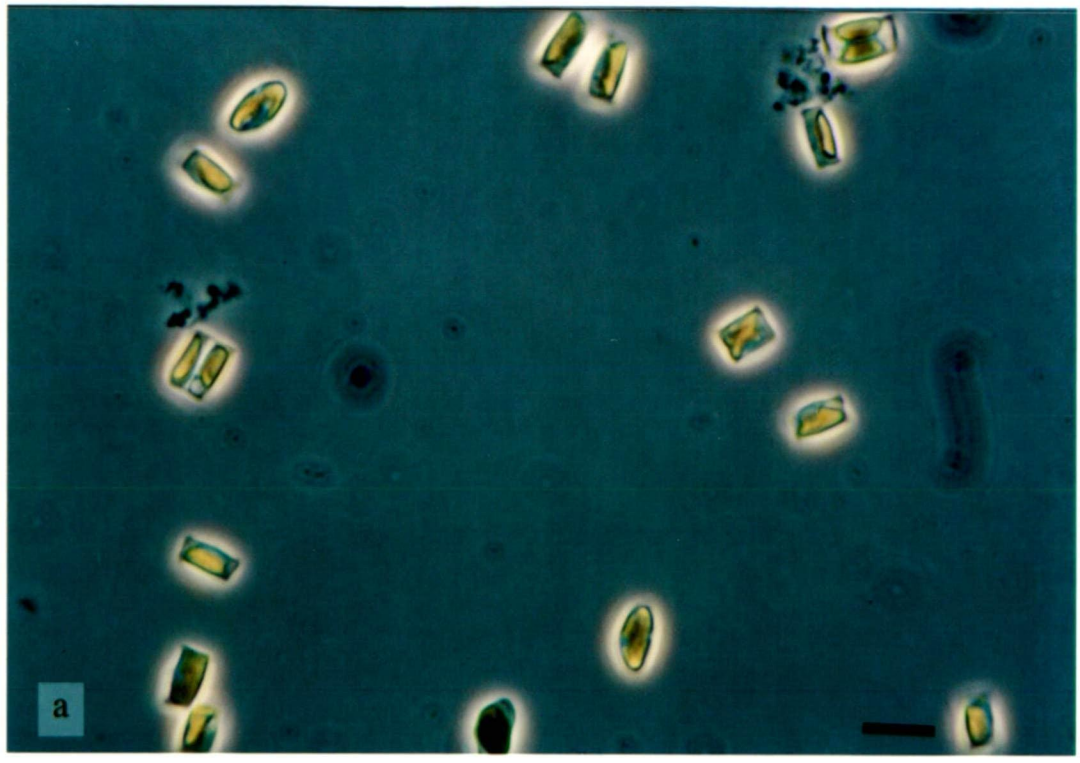
Figure 3.10 Light and transmission electron micrographs of isolate CS-431.

Papiliocellulus simplex Gardner and Crawford

Gardner and Crawford, 1992. Figures 1-10 (LM, TEM)

Fig. a (LM) Phase contrast image of live solitary cells, 8-10 µm long, 5 µm wide. **Fig b & c (TEM)** Valves were elliptical (5 µm long, 4 µm wide) consisting of a siliceous fretwork of two rows of thin transverse ribs, joined in a zigzag manner. A small annulus occurs at the centre (Fig b arrows). At the cell apices are small rimmed ocelluli (Fig c arrows). -Scale bars: 8 µm (Fig. a), 1 µm (Fig. b), 2 µm (Fig. c).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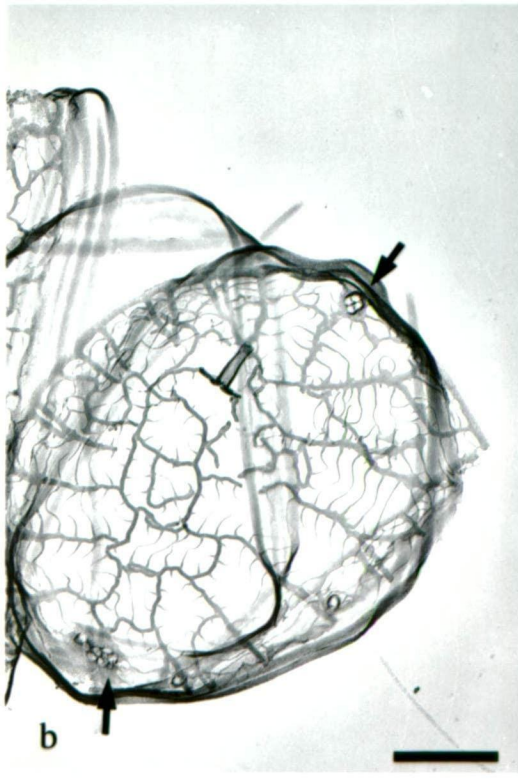
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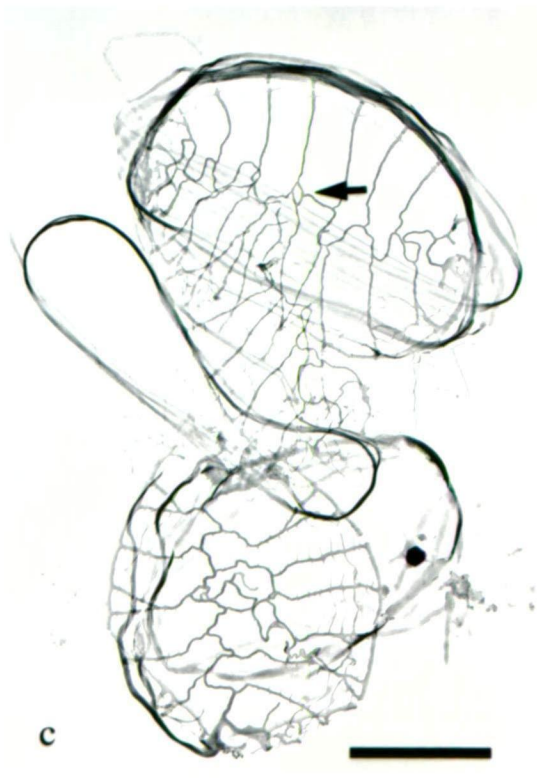

c

Figure 3.11 Light and transmission electron micrographs of isolate CS-427.

Thalassiosira oceanica Hasle

Hasle, 1983. Figures 1-8.

Fig. a & b (LM). Pairs and small colonies of live cells, circular in valve view (Fig. a, Phase contrast), and rectangular in girdle view (Fig. b, DIC -Normaski). **Fig. c & d (TEM).** Valve structure showing radial ribs (poroid areolae). One strutted process is found near the centre of the valve and a ring of distinct marginal processes with the labiate process found close one marginal process (Fig. c, arrow). -Scale bars: 5 μm (Fig. a, b), 1 μm (Fig. c), 2 μm (Fig. d).

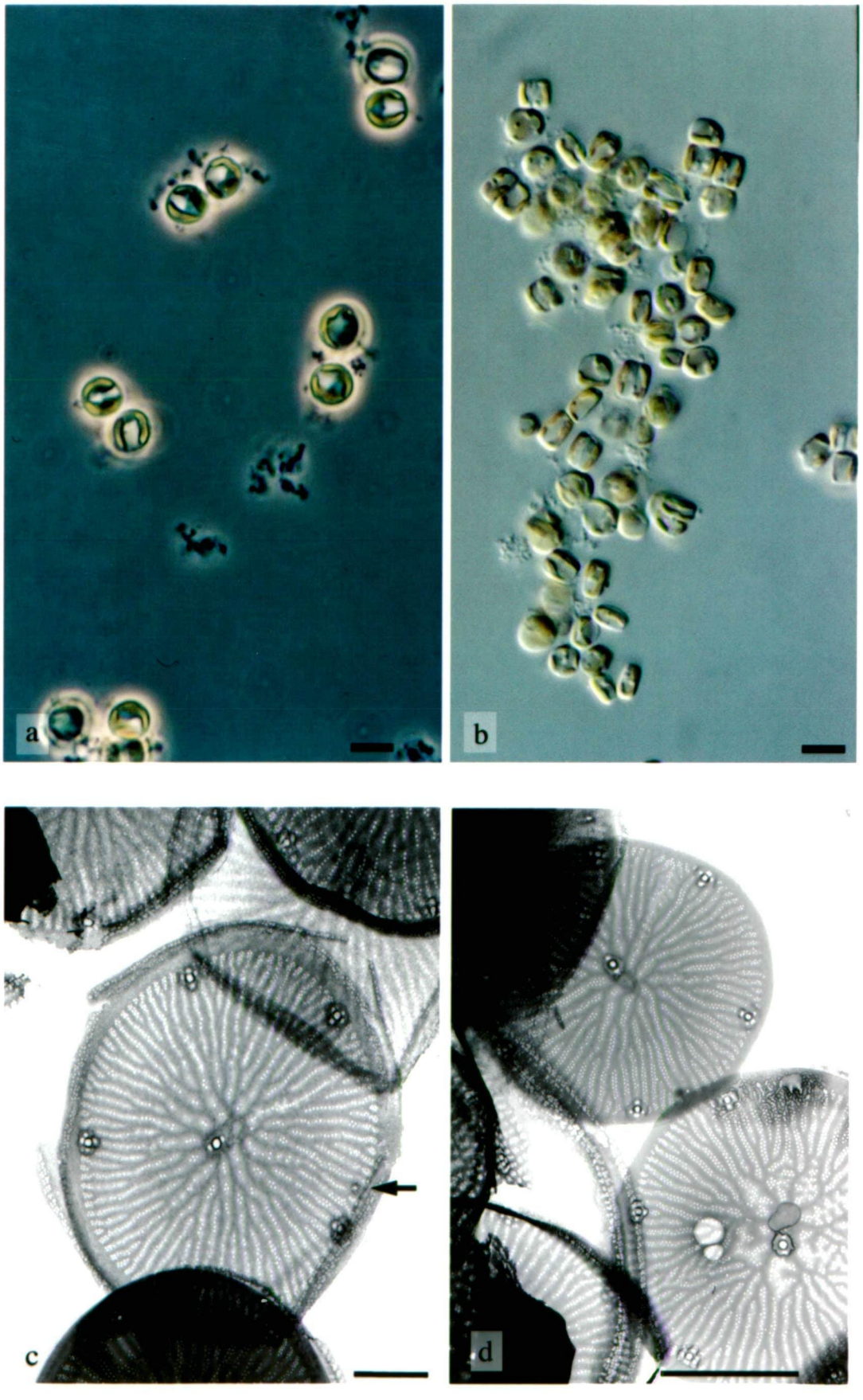
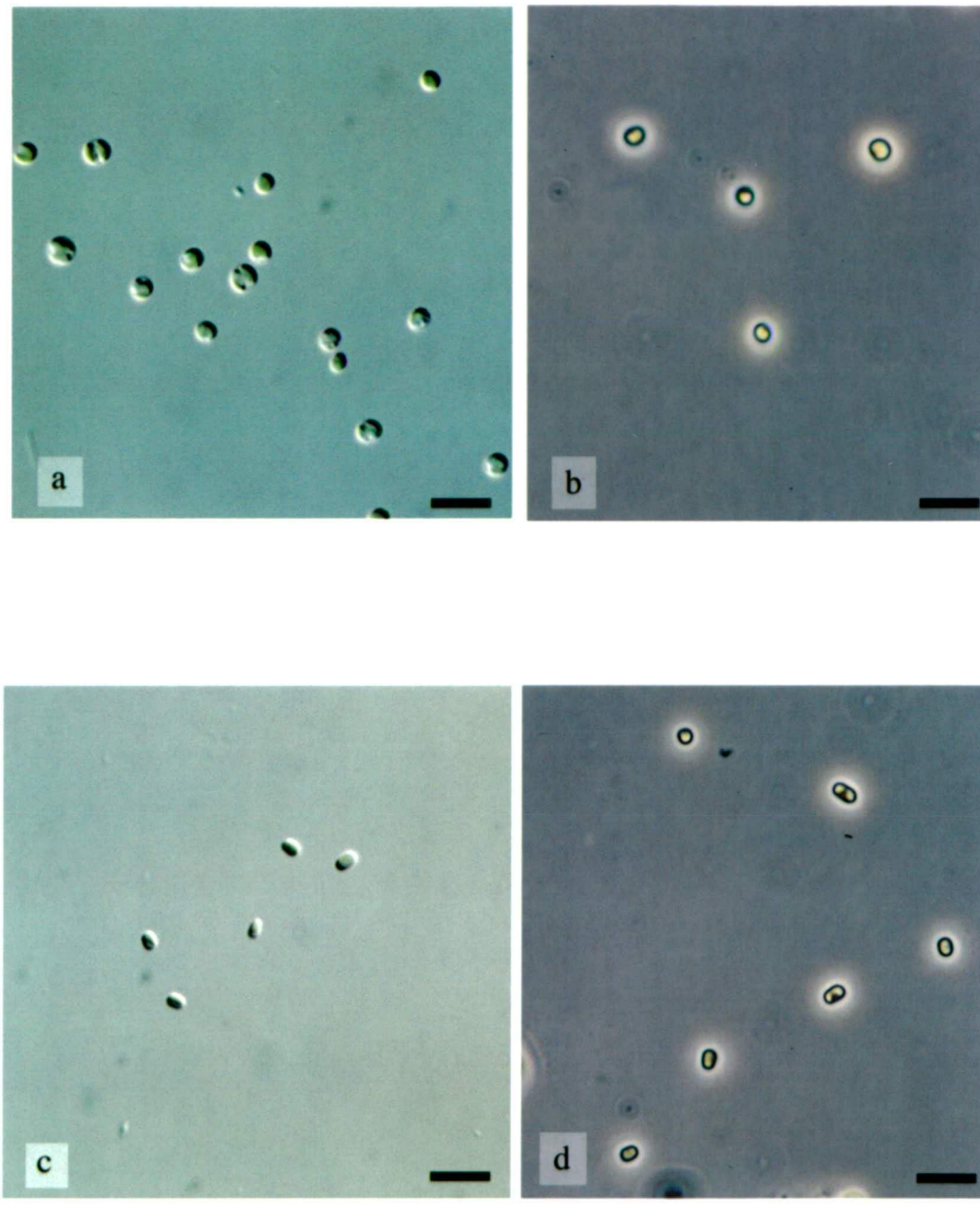

a
b
c
d

Figure 3.12 Light micrographs of chlorophyte isolates CS-436 and CS-437.

CS-436 (*Chlorella*-like)

CS-437 (*Stichococcus*-like)

Fig. a & b. Phase contrast (Fig. a) and DIC interference (Fig. b) images of isolate CS-436 showing circular cells (2 μm). **Fig. c & d.** Phase contrast (Fig. c) and DIC interference (Fig. b) images of isolate CS-437 showing oblong cells (2 μm long, 1 μm wide). -Scale bars: 5 μm (Fig. a, b, c, d).


a
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3.3 iii Growth Characteristics

The two chlorophytes were very small (2 μm CS-436, 1 x 2 μm CS-437), non-motile, single celled algae that grew to a high cell density (10^8 cells·mL^{-1}). Their cultures were stable and could be left for at least 3 months between subculturing. A complete set of growth and composition data for each species (except *Minidiscus trioculatus* CS-434) is contained in Appendix 1.

The diatom isolates included benthic (*Entomoneis* cf. *punctulata, Nitzschia* sp., *Papiliocellulus simplex*), chain forming (*Extubocellulus spinifera*), and planktonic (*Attheya septentrionalis, Minidiscus trioculatus, Thalassiosira oceanica*) species. The smallest isolates were *M. trioculatus* (3 μm) with the remainder ranging from 5-10 μm (excluding spines or chains). *Attheya septentrionalis* occurred as single or pairs of cells with spines arising at the poles of the valves and projecting parallel to the valvar plane for 2-3 times the cell length. *Extubocellulus spinifera* occurred as single cells and chains from 2-10 cells in length. In aerated, mixed cultures the chain length was shorter, rarely exceeding 4 cells. All species grew faster as aerated cultures with less clumping. Aerated cultures of the benthic *Nitzschia* (CS-429) grew rapidly (1.37 divisions·d^{-1}), with single or pairs of cells in suspension, the other *Nitzschia* isolates (CS-430, CS-433) formed suspended clumps of cells. All cultures were relatively stable and could be left for up to 8 weeks between subcultures. However, *Entomoneis* cf. *punctulata* rapidly deteriorated after this period.

Growth rates were measured as the rate of increase in cell number during exponential growth, except for the *Nitzschia* isolates CS-430 and CS-433. These isolates formed clumps that made accurate cell counts impossible and an estimation of growth rate was obtained from the rate of increase in DW (Table 3.2). Growth rates ranged from 0.55 (*Nitzschia* CS-433) to 2.04 (*A. septentrionalis*) divisions·day^{-1}. The four fastest growing isolates were the diatoms *A. septentrionalis*, *T. oceanica*, *E. spinifera*, and *Entomoneis* cf. *punctulata* with growth rates of 2.04, 1.92, 1.88, and 1.73 divisions·d^{-1} respectively. The carrying capacity of the diatom cultures were in the range of 2 - 9 x10^6 cells·mL^{-1} while the two chlorophyte isolates reached 10^8 cells·mL^{-1}. Cell mass ranged from 1 to 2 pg·cell^{-1} for the small chlorophytes to 14 to 34 pg·cell^{-1} for all diatoms except *A. septentrionalis* at 80 pg·cell^{-1}.

Table 3.2 Microalgae isolated from Tasmanian coastal waters at Little Swanport (except CS-430, Derwent R. Hobart). Results are average values from triplicate cultures. CSIRO culture numbers are those assigned to the new algal strains.

Algal Class Species	CSIRO Culture #	Axenic	Culture medium	Cell size (µm)	Cell mass (pg·cell^{-1})	Growth rate (divisions·d^{-1})	Carrying capacity (cells·mL^{-1})	Biomass (µg DW·ml^{-1})
Bacillariophyceae								
Attheya septentrionalis	CS-425	Yes	f_2	10 x 4	80	2.04	$2.1x10^6$	167
Entomoneis cf. *punctulata*	CS-426	Yes	f_2	10 x 9	34	1.73	$3.1x10^6$	114
Extubocellulus spinifera	CS-428	Yes	f_2	5 x 3	20	1.88	$7.3x10^6$	147
Thalassiosira oceanica	CS-427	Yes	f_2	5	36	1.92	$3.1x10^6$	111
Minidiscus trioculatus	CS-435	Yes	f_2	3	14	1.4	$5.8x10^6$	84
Minidiscus trioculatus	CS-432	Yes	f_2	3	16	1.05	$3.8x10^6$	70
Minidiscus trioculatus	CS-434	Yes	f_2	3	nd	nd	nd	nd
Nitzschia cf. *paleacea*	CS-433	Yes	f_2	8 x 2.5	nd	0.55*	clumping	56
Nitzschia cf. *paleacea*	CS-429	Yes	f_2	8 x 3	20	1.37	$4.9x10^6$	114
Nitzschia cf. *paleacea*	CS-430	Yes	f_2	6 x 3	nd	0.90*	clumping	130
Papiliocellulus simplex	CS-431	Yes	f_2	10x 5	26	0.66	$8.8x10^6$	180
Chlorophytes								
Isolate P1-2d (*Stichococcus* like)	CS-437	Yes	f_2	2 x 1	1	1.38	$>2.0x10^8$	279
Isolate 2e (*Chlorella* like)	CS-436	Yes	f_2	2	2	1.34	$\geq 1.4x10^8$	319

nd = not determined

* Approximate growth rate due to cell clumping (measured from rate of increase in DW).

3.3 iv. Biochemical Composition

As cultures progressed from logarithmic growth to stationary phase there were major changes in their composition (Table 3.3). Typically, the percentage ash fell sharply with a corresponding increase in one or more of the major organic fractions. In only two isolates did the ash content (% DW) remain largely unchanged: *Nitzschia* sp. CS-433, a heavily silicified benthic species, ash content fell slightly from a high 43.3% to 38.6% and the weakly silicified, *P. simplex* ash content rose slightly from a low 9.3% in logarithmic growth to 10.2% in stationary phase.

Table 3.3 Gross composition (% DW) of 1.6 L batch cultures measured during logarithmic and stationary phase of growth. Error values ± 1 S.D. (n=3).

Algal isolate	CSIRO Culture No.	Growth phase	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)	Total (%)
Attheya septentrionalis	CS-425	Log.	31.9 ± 3.7	18.0 ± 1.7	24.1 ± 0.5	26.1 ± 2.9	100
		Stat.	32.4 ± 3.0	38.5 ± 5.8	45.3 ± 2.6	8.8 ± 1.5	125
Entomoneis cf. *punctulata*	CS-426	Log.	16.9 ± 3.3	19.7 ± 4.8	28.6 ± 3.7	34.9 ± 2.9	100
		Stat.	23.5 ± 2.4	33.0 ± 3.7	24.6 ± 1.3	14.7 ± 1.3	96
Extubocellulus spinifera	CS-428	Log.	21.0 ± 1.7	15.3 ± 1.9	18.1 ± 3.3	34.1 ± 1.8	88
		Stat.	25.9 ± 1.3	22.3 ± 2.7	33.4 ± 4.2	14.0 ± 2.0	96
Thalassiosira oceanica	CS-427	Log.	14.0 ± 1.1	12.9 ± 2.4	42.3 ± 2.6	36.3 ± 2.9	105
		Stat.	17.2 ± 0.2	10.3 ± 2.1	46.0 ± 5.2	20.5 ± 3.3	94
Minidiscus trioculatus	CS-432	Log.	27.1 ± 0.9	22.0 ± 1.4	22.0 ± 1.5	29.7 ± 2.9	101
		Stat.	45.4 ± 3.7	16.6 ± 3.0	31.3 ± 3.1	24.1 ± 3.5	117
Minidiscus trioculatus	CS-435	Log.	16.7 ± 3.0	16.6 ± 3.3	17.2 ± 3.6	38.3 ± 4.6	89
		Stat.	40.6 ± 1.1	27.9 ± 0.5	23.3 ± 3.2	21.0 ± 1.9	113
Nitzschia cf. *paleacea*	CS-433	Log.	11.7 ± 1.6	9.3 ± 1.6	14.0 ± 2.0	43.3 ± 2.2	78
		Stat.	17.7 ± 3.0	7.8 ± 0.7	18.8 ± 1.3	38.6 ± 1.4	83
Nitzschia cf. *paleacea*	CS-429	Log.	21.3 ± 1.2	22.9 ± 0.9	23.5 ± 4.4	29.2 ± 2.1	97
		Stat.	41.0 ± 1.6	19.0 ± 0.2	29.9 ± 1.6	17.5 ± 1.0	107
Nitzschia cf. *paleacea*	CS-430	Log.	33.2 ± 4.6	21.1 ± 1.9	20.8 ± 4.3	22.9 ± 2.2	98
		Stat.	32.3 ± 3.9	18.4 ± 3.6	32.6 ± 0.6	14.8 ± 1.3	98
Papiliocellulus simplex	CS-431	Log.	37.8 ± 3.3	30.7 ± 0.8	27.9 ± 4.4	9.3 ± 1.0	106
		Stat.	35.1 ± 5.4	38.5 ± 5.8	20.6 ± 0.3	10.2 ± 1.0	104
Isolate 2e	CS-436	Log.	18.3 ± 1.8	17.0 ± 2.6	19.1 ± 3.3	10.5 ±1.3	65
(*Chlorella* like)		Stat.	5.0 ± 0.5	20.4 ± 1.9	22.0 ± 0.5	2.0 ± 1.0	50
Isolate P1-2d	CS-437	Log.	15.8 ± 6.5	16.7 ± 0.9	21.5 ± 2.5	19.9 ± 1.1	74
(*Stichococcus* like)		Stat.	6.7 ± 1.3	14.9 ± 1.9	17.1 ± 0.4	6.4 ± 4.9	45

Changes in the ratio of the organic fractions were variable but generally the energy storage fractions (carbohydrate and lipid) increased most in stationary phase. The percentage lipid rose in all diatoms except *Entomoneis* cf. *punctulata* and *P. simplex* and was maximal in *T. oceanica*, reaching 46% DW. Carbohydrate increased most in

A. septentrionalis and *Entomoneis* cf. *punctulata* to reach 38.5% and 33.0% DW respectively. However, percentage carbohydrate fell in *T. oceanica*, *Minidiscus* sp. CS-432 and in all *Nitzschia* isolates. Percentage protein tended to increase modestly except in *Nitzschia* sp. CS-429 and in both *Minidiscus* isolates where it increased to be the major organic fraction at over 40% DW.

3.3 iva. Lipid Analysis, Gravimetric vs Colorimetric

Gravimetric measurement of lipid in logarithmic cultures required the accurate weighing of samples of less than 1 mg. Gravimetrically determined lipid values were compared to those from a colorimetric method (section 2.3 iiib.) (Figure 3.13). On average, values from the colorimetric method were 4% lower than those obtained by gravimetric measurements. For logarithmic culture samples the difference was 6% and for stationary phase cultures 2%. The difference between logarithmic and stationary culture results indicated that logarithmic culture samples had only a slightly higher gravimetrically determined lipid value relative to stationary phase culture samples.

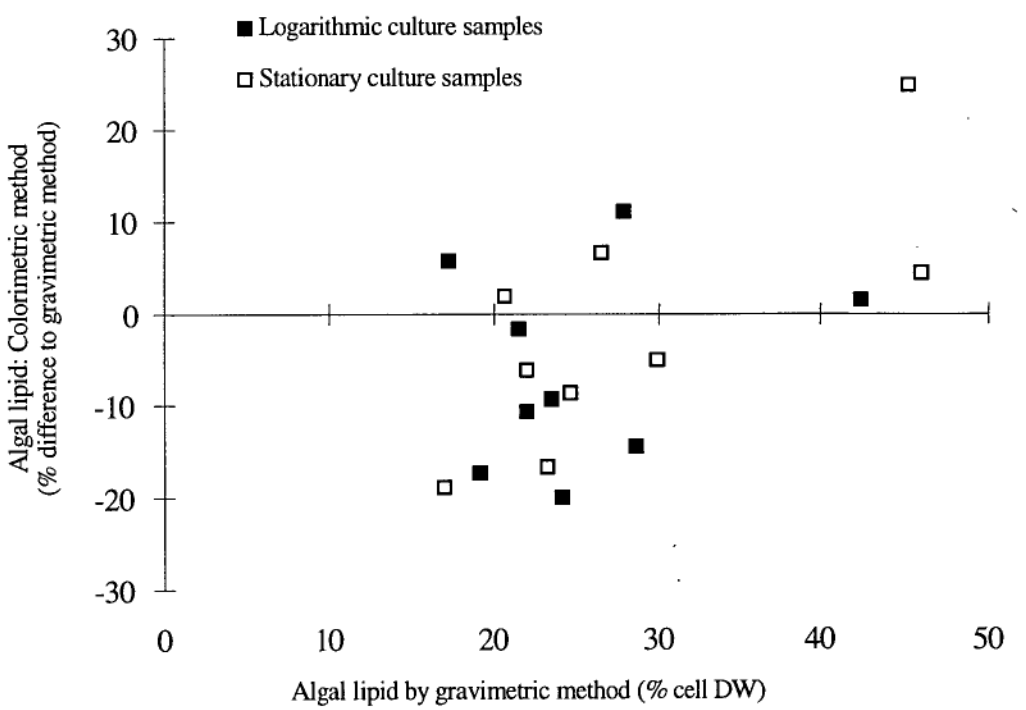


Figure 3.13 Colorimetric estimation of lipid expressed as the percentage difference to the gravimetrically determined value.

3.3 ivb. Fatty Acids

Fatty acid analysis was undertaken on the four fastest growing diatom isolates: *A. septentrionalis*, *Entomoneis* cf. *punctulata*, *E. spinifera*, *T. oceanica* (Table 3.4). A complete set of fatty acid analysis for these species is provided in Appendix 2.

As cultures went from logarithmic growth to stationary phase there was an increase in the percentage of saturated fatty acids and a decrease in mono- and polyunsaturated fatty acids (PUFA), for all isolates except *Entomoneis* cf. *punctulata*. For *Entomoneis* cf. *punctulata* cultures the fatty acid profiles remained essentially unchanged as cultures entered stationary phase. Changes in the essential oyster PUFAs, EPA (20:5n-3) and DHA (22:6n-3) are shown in Figure 3.14 (% of total fatty acids) and Figure 3.15 (% of algal DW). The major saturated fatty acids in logarithmic and stationary phase cultures were 16:0 and/or 14:0. 16:1n-7 was the major monounsaturated fatty acid (>90%).

EPA (20:5n-3), was the major PUFA in all logarithmic and stationary phase cultures (Table 3.4). It represented an average 52% of PUFAs in logarithmic cultures and 62% in stationary phase cultures. Other major PUFAs included 16:2n-7 and the other essential oyster PUFA, DHA (22:6n-3). However, DHA was not detected in *Entomoneis*

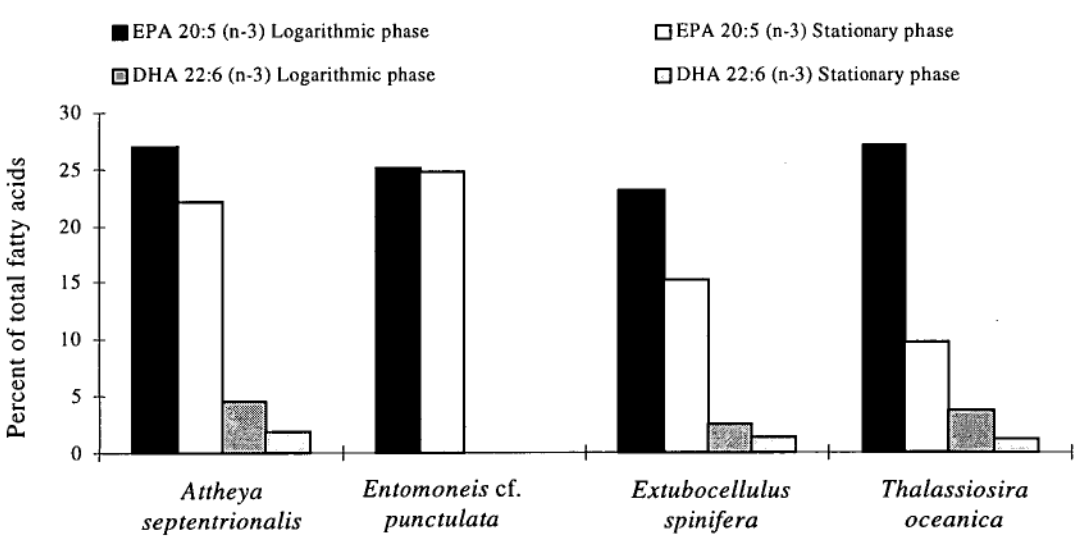


Figure 3.14 Essential PUFA content (% of total fatty acids) of four diatom isolates sampled in logarithmic and stationary phases. Averaged values, n=2.

cf. *punctulata*, but this diatom had the highest stationary phase content of another n-3 PUFA, 18:4n-3 at 5% weight of total fatty acids.

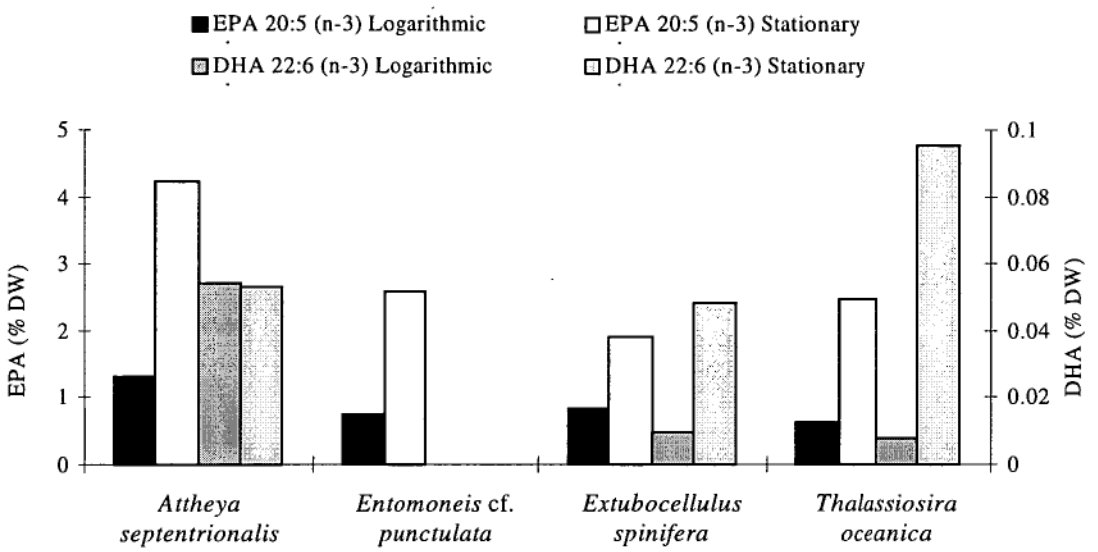


Figure 3.15 Essential PUFA content (% of algal DW) of four diatom isolates sampled in logarithmic and stationary phase. Averaged values, n=2.

Table 3.4 The fatty acid composition (% weight of total fatty acids) and total fatty acids (μg·mg^{-1} DW) of logarithmic and stationary phase cultures of four diatom isolates. Average values (n=2).

Fatty acid	*Thalassiosira oceanica*		*Entomoneis* cf. *punctulata*		*Extubocellulus spinifera*		*Attheya septentrionalis*	
	Log.	Stat.	Log.	Stat.	Log.	Stat.	Log.	Stat.
Saturates								
14:0	3.24	4.64	11.19	12.49	7.69	15.69	14.46	21.4
15:0	0.69	0.35	0.56	0.39	0.43	0.63	0.29	0.30
16:0	18.71	30.08	16.07	16.92	11.49	17.04	7.31	14.4
18:0	1.22	0.46	1.59	0.56	0.82	0.21	1.12	0.84
other	0.46	0.07	0.49	0.17	0.43	0.13	0.66	0.26
Total	24.33	35.61	29.89	30.53	20.87	33.69	23.83	37.3
Branched-chain F.A.	0.63	0.24	0.42	0.41	1.10	0.31	0.15	0.33
Monounsaturates								
16:1(n-9)	0.40	0.04	0.92	0.42	0.30	0.04	0.50	0.06
16:1(n-7)	23.65	44.75	17.98	22.89	28.17	40.41	21.50	29.1
16:1(n-5)	0.20	0.23	0.37	0.22	0.11	0.39	0.21	0.20
16:1(n-13)	0.38	0.24	0.42	0.33	0.06	0.04	0.19	0.11
18:1(n-9)	0.97	0.35	1.73	1.02	0.98	2.02	0.41	0.49
18:1(n-7)	0.36	0.28	0.32	0.15	0.38	0.18	0.84	0.25
other	0.23	0.06	0.29	0.20	0.29	0.23	0.08	0.17
Total	26.19	45.95	22.02	25.23	30.28	43.30	23.72	30.4
Polyunsaturates								
16:4(n-1)	0.38	0.07	3.87	1.45	0.37	0.11	1.98	0.52
16:2(n-7)	12.30	4.09	7.33	7.84	8.70	3.98	14.28	3.68
18:2(n-6)	0.73	0.48	1.05	0.88	0.58	0.64	1.08	0.77
18:3(n-3)	0.14	0.23	0.24	0.27	0.05	0.05	0.06	0.13
18:3(n-6)	0.07	0.00	1.91	1.41	0.40	0.21	0.66	0.37
18:4(n-3)	3.10	1.65	6.38	4.95	0.30	0.26	0.62	0.81
20:2(n-6)	0.00	0.00	0.00	0.20	0.23	0.14	0.02	0.27
20:3(n-6)	0.01	0.08	0.02	0.00	0.14	0.13	0.13	0.41
20:4(n-6)	0.00	0.00	0.00	0.00	9.85	0.00	0.00	0.00
20:4(n-3)	0.13	0.11	0.44	0.59	0.07	0.10	0.13	0.44
20:5(n-3)	27.13	9.67	25.19	24.88	23.11	15.20	26.96	22.1
22:4(n-6)	0.00	0.00	0.00	0.00	0.56	0.00	0.05	0.00
22:5(n-3)	0.01	0.00	1.08	1.29	0.07	0.04	0.07	0.07
22:5(n-6)	0.35	0.37	0.00	0.00	0.26	0.39	1.24	0.28
22:6(n-3)	3.76	1.19	0.00	0.00	2.54	1.33	4.51	1.85
other	0.05	0.01	0.03	0.02	0.01	0.01	0.01	0.00
Total	48.16	17.95	47.56	43.77	47.23	22.58	51.78	31.7
un-identified	0.69	0.26	0.11	0.06	0.52	0.12	0.53	0.11
Total (%)	100	100	100	100	100	100	100	100
Total (μg·mg^{-1} DW)	23.3	256.5	29.8	103.7	36.1	125.2	48.9	191.

3.4. *Discussion*

3.4 i. Isolation and Purification of Microalgae

Microalgal isolates were predominantly diatoms, reflecting their prevalence in the water samples and general ability to grow on solidified media. Initial, heavy bacterial contamination was inhibitory to the growth of many isolates. Selective picking of colonies and repeated streaking onto agar plates was successful in making axenic half of the final isolates while antibiotics were used for the remainder. The antibiotic combinations were chosen to give a broad antibacterial spectrum of activity. Penicillin-G is primarily effective against gram+ve bacteria while the aminoglycosides are effective against many gram+ve and most gram-ve bacteria. The bactericidal actions of penicillin and the aminoglycosides, streptomycin and kanamycin, (antibiotic combination 1) are synergistic. In combination, both the rate of killing and the minimal effective dose reflect a greater than additive action. Penicillins action in damaging the cell wall aids the entry of the aminoglycoside, and/or results in the loss of intracellular ions (Mg^{+2} and HPO_4^{-2}) that inhibit antibiotic action (Davis *et al*, 1980). The aminoglycosides neomycin and gentamicin (antibiotic combination 2) also have a broad spectrum of activity but are more toxic than those in combination 1. Chloramphenicol is relatively toxic to algae and although, like aminoglycosides in inhibiting protein synthesis, its inhibition is reversible (bacteriostatic). Since most bactericidal actions depend on some metabolic activity they are antagonised by bacteriostatic agents, therefore, chloramphenicol was used on its own as the final treatment. Several isolates were rendered axenic after exposure to antibiotic combination 1 and more after exposure to combination 2. However, chloramphenicol was ineffective in further reducing contamination and the remaining contaminated isolates were discarded. The ineffectiveness of chloramphenicol is probably due to its very low solubility in water. Viable bacteria that remained after antibiotic exposure, were predominantly non-motile, pigmented (yellow-orange-pink), gram-ve rods. Although not identified the yellow and orange colonies are likely to have been flavobacterium and the pink colonies, flectobacillus (Cropp and Garland, 1988).

3.4 ii. Identification and Growth Characteristics

HPLC pigment analysis of isolates CS-436 and CS-437 identified them as chlorophytes. Small chlorophytes are difficult to distinguish visually from eustigmatophytes, but eustigmatophytes have a specific pigment profile. They are characterised by having chlorophyll *a* as the only chlorophyll pigment and violaxanthin as the dominant xanthophyll (Patterson *et al.*, 1994). The pigment profiles of the two isolates were similar and typical of chlorophyte algae with both chlorophyll *a* and *b* present and lutein as the dominant xanthophyll (Jeffrey *et al.*, 1997).

Most diatoms were identified to species level except for the *Entomoneis* sp. and the three *Nitzschia* sp. which were tentatively identified as *E. punctulata* and *N. paleacea.* The valve structure of the *Entomoneis* sp. is similar to that of *E. punctulata,* with the striae composed of linear perforations and a short junction line (a row of basal fibulae) near the terminal corner of the wing. However, the valve length and stria density are clearly different from those of *E. punctulata* (Hallegraeff, pers. com.). *Nitzschia* can be problematic to identify (Lange-Bertalot and Simonsen, 1978) because of overlap between species in the dimensions used for identification. Isolates CS-429 and CS-430 were very similar in appearance, dimensions and biochemical composition. However, CS-433 was different visually and in composition. It was more heavily silicified and tapered more than the other blunt isolates and had a strong clumping tendency with cells of variable size. In composition it differed in having a much higher ash content and a lower carbohydrate content than either of the other *Nitzschia* isolates. It is possible that CS-433 is a different species to CS-429 and CS-430.

The diatom isolates were all of a size suitable for ingestion by oyster spat with cell masses in a range typical of other similarly sized species (Brown and Miller, 1992). Their carrying capacities of 10^6 cells·mL^{-1} were also typical of stationary phase diatom cultures grown in f/2 media. Growth rates were variable, with the four fastest growing diatoms comparable to the highly productive aquaculture species *Thalassiosira pseudonana* (2.46 divisions·d^{-1}) (Thompson and Harrison, 1992). Single or pairs of cells in culture are the best way of presenting algae to oyster spat. The *Nitzschia* isolates CS-430 and CS-433 were too clumpy to be of use as food for juvenile oysters.

The two chlorophytes were too small (1-2 pg·cell^{-1}) to be effectively ingested by oyster spat and their tough cell wall would make them indigestible (Webb and Chu, 1983). These two species have little value for the culture of oysters and limited use for other aquaculture species and were not considered for inclusion in oyster feeding experiments.

3.4 iii. Gross Composition

Microalgal isolates differed appreciably in their gross composition and in their response to stationary phase. As discussed in Chapter 1, changes in composition as cultures enter stationary phase are linked to the algal species and its response to its changing environment and in particular limitation of nutrients. As one or more nutrient becomes limiting, cultures enter stationary phase. The cell division rate falls and changes in metabolism result in the production of energy rich storage products and an increase in cell mass. Typically, with nitrogen limitation levels of lipid and/or carbohydrate rise (Fig. 1.3; Harrison *et al.*, 1990; Enright *et al*, 1986b) and protein levels fall (Utting, 1985; Kreeger and Langdon, 1993). In silicate-limited *Chaetoceros gracilis* cells, protein levels remained unchanged while the lipid content more than doubled and carbohydrate increased 1.6 fold (Enright *et al*, 1986b). In evaluating algae, they were grown under conditions similar to those they would encounter in an aquaculture facility using a typical minimal nutrient media such as f/2. The changes in composition are therefore, those that are likely to occur during mass culture of the algae if used for aquaculture feeds.

For the diatom isolates protein and lipid were more frequently the dominant fraction. This is in agreement with Brown *et al's.*, (1997) results for a range of diatoms (Fig. 1.1) although, average values and ranges for lipid and carbohydrate were greater than theirs. Greater carbohydrate levels could be a result of using continuous illumination, which can more than double the carbohydrate content (Fig. 1.2). *Thalassiosira oceanica* had a low carbohydrate content which fell in stationary phase, a similar effect is reported for *Thalassiosira pseudonana* as a likely function of glycolytic action for energy following very rapid growth (Whyte, 1987).

The sum of analysed components for the diatoms was generally close to 100% (average for logarithmic cultures = 96%; stationary phase cultures = 103%), but was very low (~50%) for stationary phase cultures of the two chlorophyte species. Their tough cell walls resisted extraction and solubilisation, recovery of protein appeared very low in these culture samples. Carbohydrate was over-estimated in stationary phase cultures of *A. septentrionalis* and *Entomoneis* cf. *punctulata*, if samples were not firstly solvent extracted. This effect was not found in logarithmic cultures and may be due to interference from lipids since their removal by solvent extraction reduced measured carbohydrate. Gravimetrically determined lipid values for logarithmic phase cultures involved weighing lipid extracts of <1 mg. Accuracy was confirmed by comparing results to the colorimetric, dichromate assay (section 3.3 iva.) which indicated only a slight over-estimation of lipid compared to larger weight samples.

Phytoplankton rich in carbohydrate and lipid are reported to support faster oyster growth (Flaak & Epifanio, 1978; Enright *et al.*, 1986a; Wikfors *et al.*, 1996). However, aquaculture algae must have a rapid growth rate to maximise production and minimise costs. The four isolates chosen for assessment as feed for juvenile *Crassostrea gigas* all had rapid growth rates (>1.5 division·d^{-1}) and accumulated carbohydrate and/or lipid in stationary phase. They were *Attheya septentrionalis*, *Entomoneis* cf. *punctulata*, *Extubocellulus spinifera,* and *Thalassiosira oceanica.*

Although the PUFAs 20:5n-3 and 22:6n-3 have been shown to be essential for oysters (Langdon and Waldock, 1981), the exact diet requirement is unknown. Fatty acid analysis of the selected algae showed them all to be a rich source of the essential PUFA 20:5n-3. However, *Entomoneis* cf. *punctulata* was deficient in the other essential PUFA, 22:6n-3 and might not be suitable for use as a single species diet. Incorporating it in a mixed diet containing algae rich in 22:6n-3 would compensate for the deficiency.

In chapter 4, the nutritional value to juvenile Pacific oysters of the four selected species is assessed. They were fed as part of ternary diets, which included the test diatom and a minor component of the prymnesiophytes, *Isochrysis* sp. (T. iso) and *Pavlova lutheri.* This experimental design compensates for nutritional deficiencies such as that

mentioned for *Entomoneis* cf. *punctulata* and allows for assessment of the diets based on their gross composition.

Chapter Four

Assessment of Australian Isolates as Oyster Feed

Chapter 4

Assessment of Australian Isolates as Oyster Feed

4. 1 *Introduction*

In chapter 3, four diatom isolates (*Attheya septentrionalis*, *Entomoneis* cf. *punctulata*, *Extubocellulus spinifera*, and *Thalassiosira oceanica*) were selected as potential feed species for juvenile oysters. Selection was based on isolates having a high productivity and a composition rich in lipid and carbohydrate.

4.1 i. Nutritional Value of Diatom Isolates for Juvenile Pacific Oysters

The exact nutritional requirements of bivalves are poorly defined, partly because of the lack of artificial diets. However, through feeding trials with algae of partially defined compositions, trends have emerged. Carbohydrate and n-3 PUFA levels in phytoplankton species were identified as major factors in a good algal diet for *Ostrea edulis* (Enright *et al.*, 1986a). Microalgae with high concentrations of carbohydrate were found to give the best oyster growth, providing adequate protein and essential fatty acids were supplied (Enright *et al.*, 1986b). Supplementing juvenile mussel (*Mytilus trossulus*) diets with protein microcapsules gave a positive growth response to the increase in dietary protein and indicated a protein content below 40% w/w can reduce mussel growth rates (Kreeger and Langdon, 1993).

The nutritional value of an alga is related to its ability to supply essential micronutrients to consumer organisms and by its gross composition, which is dependent on a wide range of factors as outlined in Chapter 1. Mixed algal diets are better able to continually supply a consumer's dietary requirement and outperform monoalgal diets (Epifanio, 1979, 1982, Webb and Chu, 1982). Because of this they are routinely used in oyster cultivation (Coutteau and Sorgeloos, 1992). Diatoms and prymnesiophytes are nutritious algae that are widely used as feeds for oysters (Coutteau and Sorgeloos, 1992). The prymnesiophytes *Isochrysis* sp. (T. iso) and *Pavlova lutheri* are rich sources of 22:6n-3 (8-10% of total fatty acid fraction) (Volkman *et al.*, 1989), while diatoms are a rich source of 20:5n-3 (Dunstan *et al.*, 1994). A mixed algal diet of prymnesiophytes

and diatoms would therefore satisfy requirements for essential PUFAs and be highly nutritious for bivalve molluscs.

To evaluate the diatom isolates they were fed as part of a ternary diet to juvenile Pacific oysters (*Crassostrea gigas*). The ternary diets contained a diatom species with a binary diet of equal quantities of *Isochrysis* sp. (T. iso) and *Pavlova lutheri.* The binary diet was the minor component at a maximum of 30% of total dietary dry weight. Keeping the prymnesiophyte component constant while varying the diatom species maintained the algal class composition of the diet. This reduces differences in diet performance that can result from peculiarities of algal class, thus leaving differences in performance due mostly to variation in the gross composition of the diets. The four diatom isolates were compared to a reference diatom, *Thalassiosira pseudonana.* Effect of the diets on the composition and growth of the oysters is discussed in relation to the diets' biochemical composition and the nutritional requirements of the oyster.

4.2. *Materials and Methods*

4.2 i. Oyster Culture

Juvenile oysters were provided by Shellfish Culture Ltd. and the feeding trial conducted at their nursery at Pipe Clay Lagoon, 30 km Southeast of Hobart, Australia (42° 58' S, 147° 32' E). For oyster culture, scaled down (1/20th) models of commercial upwellers were used (section 2.1 ii.). Freshly graded oysters (1 mL of 1500 μm size) were added to each chamber and the buckets filled with 8 L of 0.6 μm filtered seawater. Four randomly distributed replicates of each diet were tested. Oysters were allowed to acclimatise for two days before feeding. Buckets and chambers were cleaned daily with a fine spray of 0.6 μm filtered seawater and refilled with the filtered seawater. Temperature was maintained at 18-20 °C.

At the start of the experiment five samples (1 mL of 1500 μm size) of oysters were collected as 'day 0' samples. They were rinsed (3 x 10 mL) in Milli-Q water and a subsample of 50 oysters were counted from each sample. Dry weights (DW) and ash free dry weights (AFDW) were determined (section 2.1 ii.) and the weight of the subsample of oysters was used to estimate the total number of oysters and the individual oyster weight. Residual, freshly graded oysters left over after removal of experimental replicates (28 x 1 mL for experimental treatment and 5 x 1 mL samples for 'day 0' measurements) were returned to their nursery upweller. These oysters received continuous flow-through seawater containing natural phytoplankton. They were removed from the nursery's routine grading protocol and were sampled at completion of the feeding experiment (4 x 1 mL) to compare the growth of experimental oysters to oysters maintained in the commercial nursery.

At completion of the feeding experiment, oysters from each chamber were rinsed (3 x 10 mL) in Milli-Q water and 50 oysters removed for biochemical analysis. The remainder were dried and ashed to determine DW and AFDW. Oyster growth rates were determined from measurements of the initial and final DW and AFDW to give an instantaneous growth rate (k) in d^{-1} (section 2.1 ii.).

Apparent growth efficiency (%AGE) was determined for each diet according to the equation:

$$\%AGE = AFDW_{incr} / AFDW_{diet}$$

where $AFDW_{incr}$ is the increase in the AFDW of the oysters and the $AFDW_{diet}$ is the total AFDW of the fed algal diet. The efficiency of use by the oysters of the dietary components was estimated following an expression adapted from Albentosa *et al.*, (1996):

$$CRI = (C_f - C_i) / C_{fed}$$

where CRI is the retention index for each component, CRI(protein), CRI(lipid) and CRI(carbohydrate); C_i and C_f are the contents for each of the major components in the oysters at the beginning and end of the experiment, and C_{fed} is the quantity of dietary component fed during the experiment.

4.2 ii. Microalgae, Diets and Analysis

The test diatom species, i.e. *Attheya septentrionalis*, *Entomoneis* cf. *punctulata*, *Extubocellulus spinifera*, and *Thalassiosira oceanica*, (Figure 4.1 - 4.4), were isolated and selected as outlined in Chapter 3. *Thalassiosira pseudonana* (CS-173), *Isochrysis* sp. (T. iso) (CS-177) and *Pavlova lutheri* (CS-182) were obtained from the CSIRO Collection of Living Microalgae, Division of Marine Science, Hobart, Tasmania.

Algae were grown in 10 L polycarbonate carboys (section 2.1 i.) and maintained in late-exponential phase by replacing 20% of the volume every 2 days with fresh media. A new culture was used each week so that three separate 10 L cultures of each algal species was used during the trial. A pretrial established that 1 mL of oysters (1500 μm size) fed 10 mg DW of algae, consumed over 80% of the algae over 24 h. This feed rate (10 mg DW·bucket $^{-1}$·d^{-1}) was used for the first nine days then increased by 30 % to 13 mg from day 10-15 and to 17 mg from day 16-21. Throughout the experiment both prymnesiophytes were added at 1.5 mg DW·d^{-1} with only the diatom component increasing. At each increase in feed rate, algal cell counts before and after feeding confirmed the minimum consumption to be over 80%. Daily feed volumes were

determined from DW and AFDW measurements. Daily subsamples (10-20 mL) of each algal diet were filtered through 25 mm glass-fibre filters for gross composition analysis. Filters were stored at -20 °C and analysed within 6 months.

Before analysis, the filtered algal samples taken daily during the experiment were grouped to form three composite samples for each algal species. The composite samples representing the period that each of the three 10 L carboy cultures, used during the experiment, were fed to the oyster spat. The subsamples of 50 oysters were ground with a mortar and pestle. Algal and oyster samples were then analysed for lipid, protein, mono and oligo-saccharides and polysaccharide after fractionation of the samples (section 2.2.).

The total dietary energy (J) fed to experimental replicates was estimated using the calorific equivalents for protein, carbohydrate and lipid of 20.1, 17.6 and 39.7 $kJ \cdot g^{-1}$, respectively (Whyte, 1987).

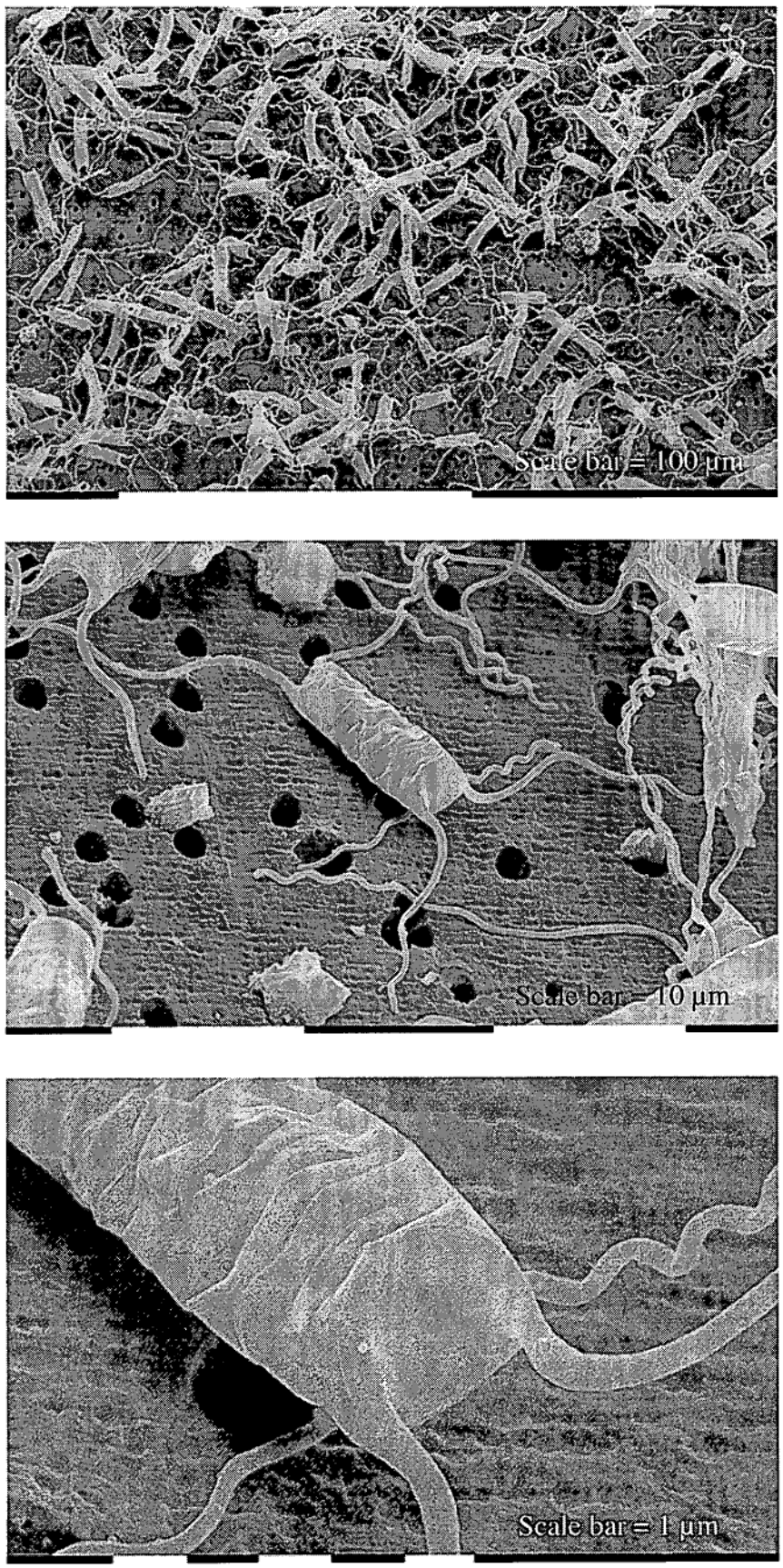


Figure 4.1 SEM images of *Attheya septentrionalis*

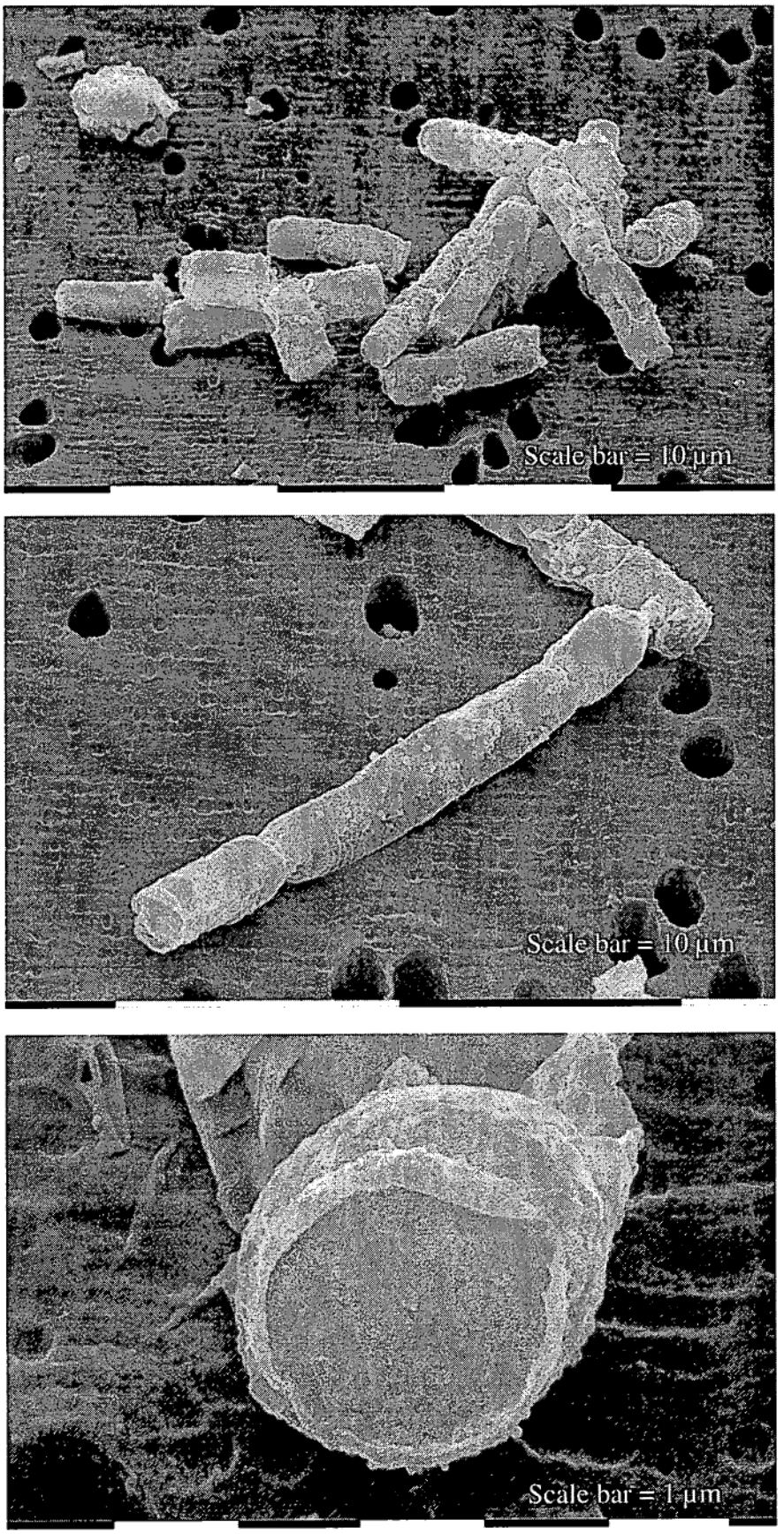


Figure 4.2 SEM images of *Extubocellulus spinifera*

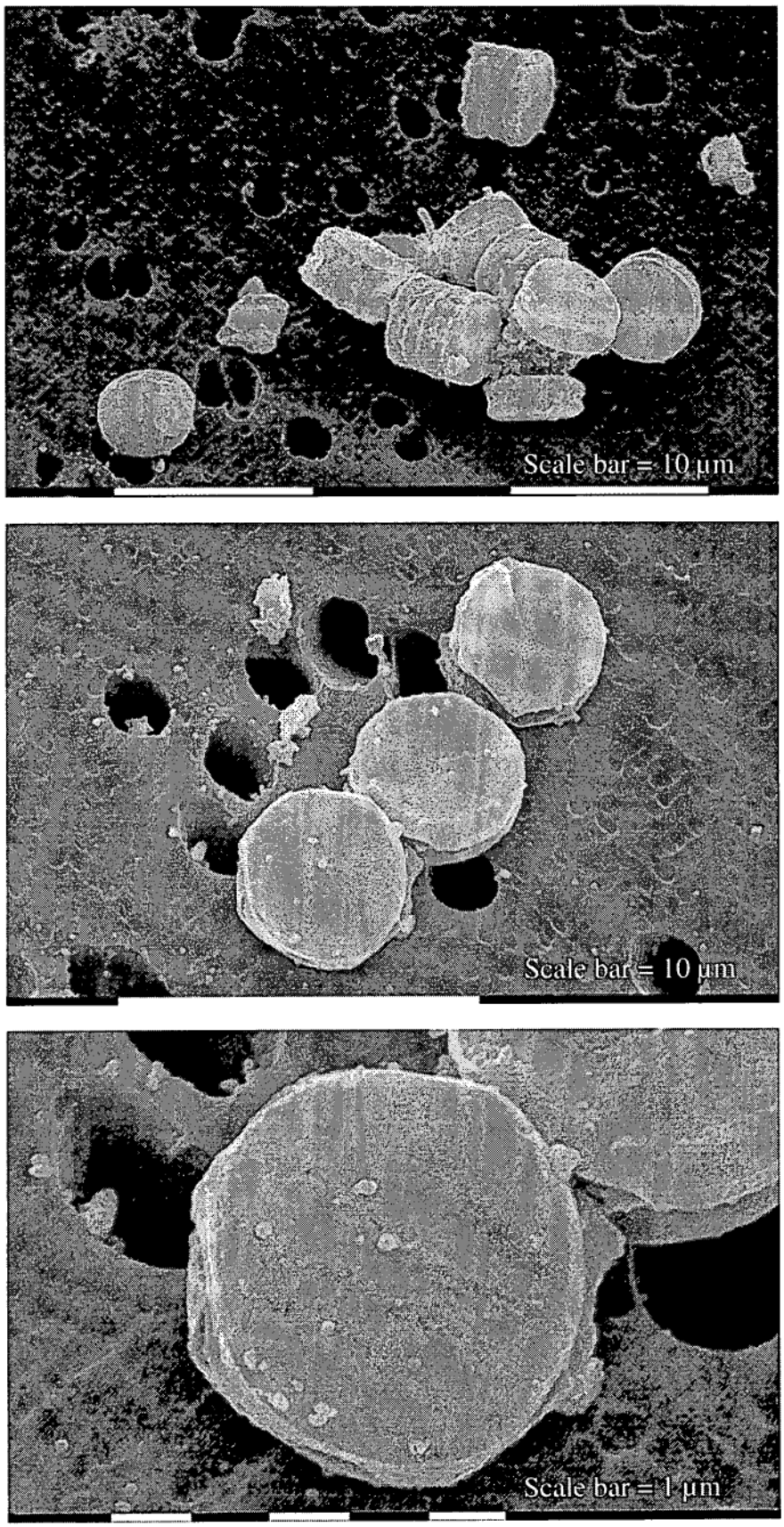
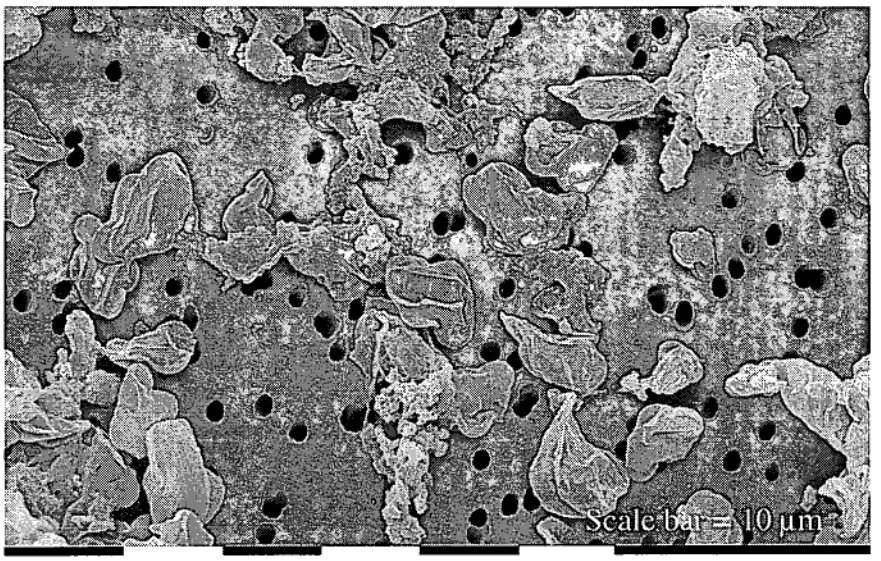


Figure 4.3 SEM images of *Thalassiosira oceanica*



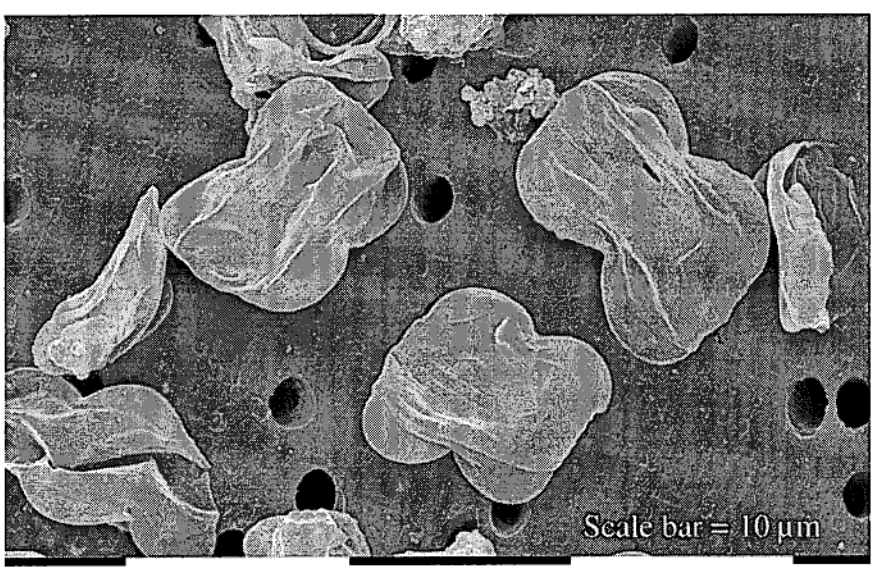


Figure 4.4 SEM images of *Entomoneis* cf. *punctulata*

4.3. *Results*

4.3 i. Gross Composition of Algal Cultures and Diets

Gross analysis of the semicontinuously harvested algal feed cultures showed the four test diatoms to have a composition in between that of logarithmic and stationary phase batch cultures (Table 4.1).

Table 4.1 Gross composition (% DW) of semicontinuously harvested algal feed cultures and comparison to 1.6 L batch cultures (from Table 3.3) measured during logarithmic and stationary phase of growth. Error values ± 1 S.D. (n=3).

Algal isolate CSIRO Culture No.	Growth phase	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)	Total (%)
Attheya septentrionalis	Log.	31.9 ± 3.7	18.0 ± 1.7	24.1 ± 0.5	26.1 ± 2.9	100
CS-425	Stat.	32.4 ± 3.0	38.5 ± 5.8	45.3 ± 2.6	8.8 ± 1.5	125
	Semi	22.1 ±4.2	23.9 ±7.0	35.3 ±9.4	13.9 ±2.8	95
Entomoneis cf. *punctulata*	Log.	16.9 ± 3.3	19.7 ± 4.8	28.6 ± 3.7	34.9 ± 2.9	100
CS-426	Stat.	23.5 ± 2.4	33.0 ± 3.7	24.6 ± 1.3	14.7 ± 1.3	96
	Semi	26.3 ±3.7	28.4 ±5.1	27.7 ±9.8	17.8 ±4.7	100
Extubocellulus spinifera	Log.	21.0 ± 1.7	15.3 ± 1.9	18.1 ± 3.3	34.1 ± 1.8	88
CS-428	Stat.	25.9 ± 1.3	22.3 ± 2.7	33.4 ± 4.2	14.0 ± 2.0	96
	Semi	19.0 ±2.0	10.4 ±1.0	25.5 ±7.3	21.3 ±3.6	76
Thalassiosira oceanica	Log.	14.0 ± 1.1	12.9 ± 2.4	42.3 ± 2.6	36.3 ± 2.9	105
CS-427	Stat.	17.2 ± 0.2	10.3 ± 2.1	46.0 ± 5.2	20.5 ± 3.3	94
	Semi	18.1 ±1.3	8.2 ±1.4	39.9 ±11.0	18.8 ±4.0	85
T. pseudonana	Semi	28.0 ± 1.8	15.1 ±2.6	31.6 ± 6.6	12.9 ± 2.8	87
CS-173						
Isochrysis sp. (T. iso)	Semi	21.5 ± 1.3	26.7 ±2.7	31.9 ± 1.8	5.0 ± 2.0	85
CS-177						
Pavlova lutheri	Semi	28.8 ± 3.5	11.7 ±1.6	37.0 ± 4.4	4.9 ± 1.3	82
CS-182						

The ternary diets (ie, the binary diet of *Isochrysis* sp. (T. iso) and *Pavlova lutheri*, with one of five diatoms) were all fed at a similar total DW and AFDW with total amounts of 274 ± 5 mg and 234 ± 5 mg respectively (Table 4.2). The ternary diets differed in

their composition by virtue of the difference in the composition of the diatoms making up the diet (Table 4.2). Diets containing *Attheya septentrionalis* and *Entomoneis* cf. *punctulata* contained the most carbohydrate (24.4 and 25.4% DW respectively), *Entomoneis* cf. *punctulata* and *Thalassiosira pseudonana* contained the most protein (25.6 and 25.8% DW respectively) and *Thalassiosira oceanica* contained the most lipid (35.3% DW). *Extubocellulus spinifera* had a high ash content which, combined with a lower percentage of gross components (76 % of DW), resulted in the lowest total dietary energy available from gross components (4581 Joules), compared to 5460-5826 J for other ternary diets. Cell counts, prior and post feeding, showed the minimum consumption to be >80% and typically >90%.

Table 4.2 Gross composition and energy content of algal diets fed to *Crassostrea gigas* spat. Diet compositions shown as total milligrams fed over the duration of the trial and as percentage algal dry weight (% DW).

		Binary diet		Binary diet +									
	Unfed control	*Isochrysis* sp. (T. iso) + P. *lutheri*		*Extubocellulus spinifera*		*Thalassiosira oceanica*		*Attheya septentrionalis*		*Entomoneis* cf. *punctulata*		*Thalassiosira pseudonana*	
Diet gross composition		(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)
Protein		13.5	21.2	53.0	19.1	51.7	18.7	61.8	23.2	71.0	25.6	70.1	25.8
Lipid		22.3	34.9	73.4	26.4	97.5	35.3	86.6	32.5	76.4	27.6	82.5	30.4
Polysaccharide		11.2	17.6	25.8	9.3	21.2	7.7	30.7	11.5	38.7	14.0	25.3	9.3
Mono- oligosaccharides		1.3	2.1	8.4	3.0	10.1	3.6	34.3	12.9	31.6	11.4	20.2	7.4
Total carbohydrate		12.5	19.6	34.1	12.3	31.3	11.3	65.0	24.4	70.3	25.4	45.5	16.7
Ash		3.3	5.1	50.5	18.2	44.3	16.0	31.7	11.9	42.3	15.3	30.0	11.0
Total gross components		52	81	211	76	225	81	245	92	260	94	228	84
Total dietary algae (DW)	0	64	100	278	100	276	100	267	100	277	100	272	100
Dietary energy		(J)	(%)	(J)	(%)	(J)	(%)	(J)	(%)	(J)	(%)	(J)	(%)
Energy from protein		272	20	1064	23	1039	19	1243	21	1428	25	1409	26
Energy from lipid		885	64	2915	64	3870	71	3439	59	3033	53	3274	60
Energy from carbohydrate		221	16	601	13	551	10	1144	20	1236	22	801	15
Total dietary energy	0	1378	100	4581	100	5460	100	5826	100	5697	100	5484	100

4.3 ii Growth and Gross Composition of Experimental Oysters

Over the 21 d experimental period, the specific growth rates of oysters, k (determined from AFDW), were all significantly ($P<0.05$) different except for those fed diets containing *Attheya septentrionalis* and *Entomoneis* cf. *punctulata* (Figure 4.5). The ternary diet containing *Thalassiosira pseudonana* produced a significantly faster growth rate ($k= 0.055\ d^{-1}$, $P<0.05$). The performance of the ternary diet containing *Attheya septentrionalis* was equal to that containing *Entomoneis* cf. *punctulata* ($k= 0.050\ d^{-1}$), and both outperformed diets containing *Thalassiosira oceanica* and *Extubocellulus spinifera* ($k= 0.041\ d^{-1}$, $k= 0.036\ d^{-1}$, respectively). All ternary diets outperformed the binary diet ($k= 0.015\ d^{-1}$) and the unfed control oysters ($k= -0.014\ d^{-1}$) which lost AFDW (organic content). Oyster DW showed similar trends except that the unfed control oysters showed a small weight gain (14%) from shell growth (Table 4.3).

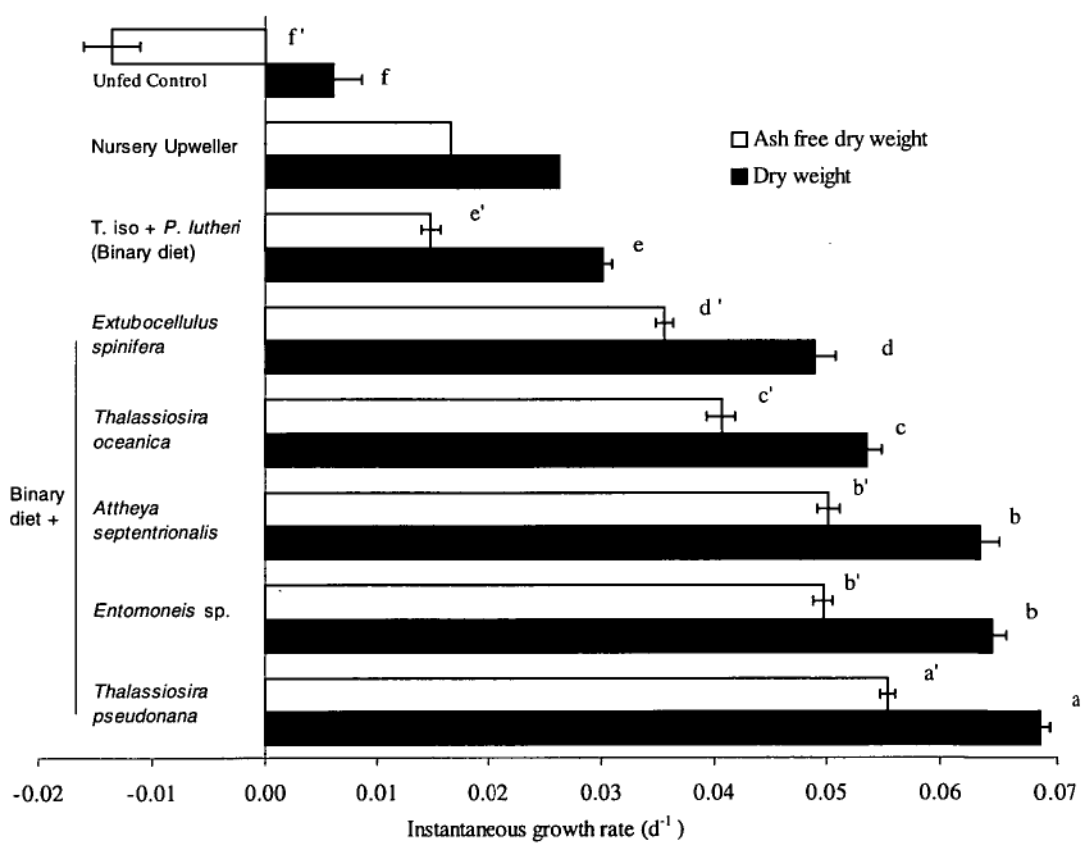


Figure 4.5 Oyster instantaneous growth rates when fed a range of experimental algal diets and comparison to growth rates achieved with commercial production (nursery upweller). Bars without a common letter are significantly different ($P<0.05$). Error bars are ± 1 S.D. (n=4).

Table 4.3 Gross composition (% AFDW), growth and apparent growth efficiency (%) of juvenile oysters fed a range of algal diets. Error values are ± 1 S.D. (n=4).

				Binary diet	Binary diet +				
	Initial oysters	Nursery upweller	Unfed control	= (T. iso + *P. lutheri*)	*Extubocellulus spinifera*	*Thalassiosira oceanica*	*Attheya septentrionalis*	*Entomoneis* cf. *punctulata*	*Thalassiosira pseudonana*
Oyster gross composition (% AFDW)									
Protein	41.46 ±2.57	39.94 ±4.73	54.66 ±6.68	47.29 ±5.75	46.03 ±4.13	46.96 ±2.67	49.31 ±5.29	58.63 ±4.20	52.33 ±2.76
Lipid	14.19 ±1.51	16.96 ±1.54	11.91 ±1.99	13.00 ±2.17	17.28 ±1.54	16.40 ±1.08	15.01 ±0.87	15.91 ±0.72	13.88 ±0.44
Polysaccharide	4.94 ±0.16	4.18 ±0.62	3.82 ±0.28	3.97 ±0.54	4.53 ±0.44	4.21 ±0.34	4.38 ±0.14	4.58 ±0.15	4.31 ±0.14
Mono- oligosaccharides	2.74 ±0.13	1.63 ±0.28	1.32 ±0.10	1.67 ±0.12	1.86 ±0.17	1.85 ±0.08	2.26 ±0.31	2.87 ±0.39	2.07 ±0.16
Total carbohydrate	7.67 ±0.10	5.81 ±0.78	5.14 ±0.30	5.64 ±0.61	6.39 ±0.54	6.06 ±0.27	6.64 ±0.40	7.45 ±0.29	6.38 ±0.13
Total gross components	63	63	72	66	70	69	71	82	73
Oysters (277 ±13 oysters/replicate)									
DW (mg ·oyster^{-1})	1.26 ±0.03	2.19	1.43 ±0.08	2.37 ±0.04	3.52 ±0.14	3.88 ±0.11	4.78 ±0.17	4.89 ±0.13	5.33 ±0.09
AFDW (µg ·oyster^{-1})	113 ±2	160	85 ±5	154 ±3	238 ±4	265 ±7	323 ±7	320 ±6	362 ±5
Percentage increase in DW		74	14 ±6	88 ±4	179 ±7	208 ±11	279 ±9	288 ±13	323 ±11
Percentage increase in AFDW		42	-25 ±4	36 ±3	111 ±5	135 ±4	187 ±6	184 ±6	221 ±5
Total diet AFDW (mg)			0	61	227	232	235	235	242
Total increase in oyster AFDW (mg)				11	35	42	58	57	69
Apparent growth efficiency (%)				19	15	18	25	24	29

Stepwise regression on all treatments (N=7) showed dietary protein to be the most significant component effecting oyster growth (Figure 4.6). Similar analysis on only the ternary diets (N=5) also showed dietary protein to be the single most significant component and dietary energy as the best alternative to protein.

Stepwise regression on all 7 treatments:

N=7 Weight increase ($\mu g \cdot oyster^{-1}$) = -22.32 + 0.962* dietary protein ($\mu g \cdot oyster^{-1}$)

$r^2 = 0.949$ r^2 (adjusted) = 0.939 P = <0.0005

Stepwise regression on the 5 ternary diets:

N=5 Weight increase ($\mu g \cdot oyster^{-1}$) = -106.05 + 1.33* dietary protein ($\mu g \cdot oyster^{-1}$)

$r^2 = 0.783$ r^2 (adjusted) = 0.711 P = <0.05

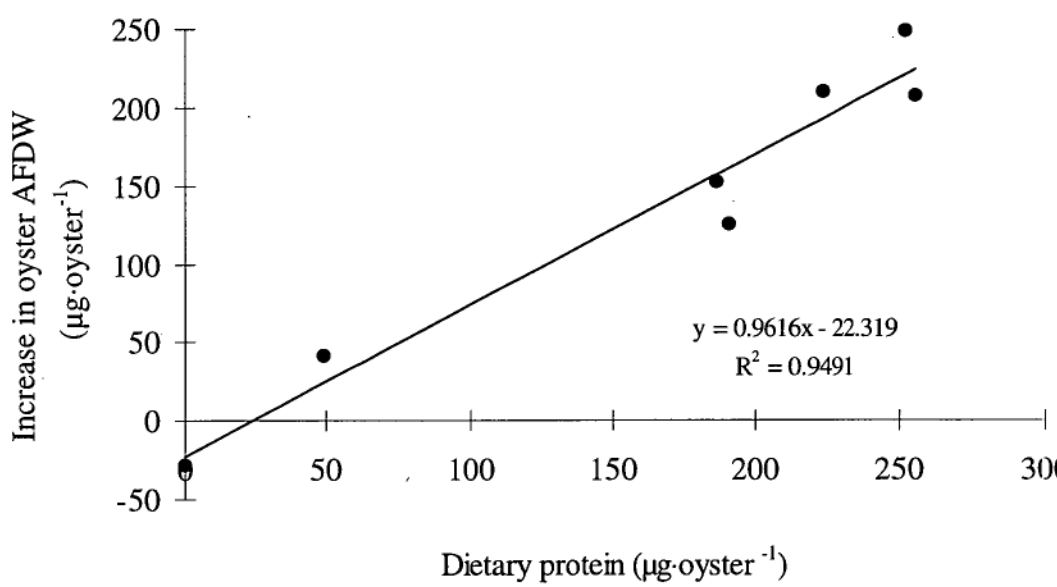


Figure 4.6 Relationship between dietary protein ($\mu g \cdot oyster^{-1}$) and oyster growth (μg AFDW$\cdot oyster^{-1}$) for all seven experimental treatments.

The protein component retention index, CRI (protein) for oysters fed the ternary diets containing *Thalassiosira pseudonana, Attheya septentrionalis* or *Entomoneis* cf. *punctulata* ranged from 0.50-0.56 showing protein to be highly conserved (Figure 4.7). The ternary diets that produced the least oyster growth contained *Thalassiosira oceanica* or *Extubocellulus spinifera* and these also had a lower CRI (protein) of 0.42

and 0.33, respectively. Protein was also conserved in oysters fed the binary diet (CRI of 0.53) and protein levels remained static in the unfed oysters.

In contrast to protein, the CRI for carbohydrate and lipid showed them to be poorly conserved. Oysters fed the ternary diets had similar low CRI (carbohydrate) values (0.05-0.09). For those fed the binary diet the CRI (carbohydrate) was 0 indicating no overall increase in oyster carbohydrate associated with growth and therefore a fall in the percentage carbohydrate of the oysters. The unfed control oysters lost 48% of their initial carbohydrate content. Lipid was also poorly conserved with CRI (lipid) values ranging from 0.08-0.12 for the ternary diets to only 0.03 for the binary diet. The unfed control oysters lost 37% of their lipid content.

The gross composition of the diets affected the gross composition of the juvenile oysters. The influence of diet composition on oyster composition is best determined by analysing only the five ternary diets which had different gross compositions but were fed at an equal algal and energy ration. The strongest correlation was seen when comparing the percentage carbohydrate of oyster to the total carbohydrate fed in the diets (Figure 4.8). An ANOVA on the ternary diets showed a significant diet effect ($F=8.85$, $df=4, 15$, $P=0.001$) of carbohydrate on the percentage carbohydrate content of oyster tissue. Partitioning the diet effect showed a significant linear effect due to carbohydrate content in the diets ($F=26.39$, $df=1, 15$, $P<0.001$) and no significant deviations from linear ($F=0.20$, $df=5, 21$, $P=0.89$).

Total dietary protein also effected the final percentage protein levels in the oysters. An ANOVA showing a significant diet effect ($F=6.68$, $df=4, 15$, $P=0.003$) of protein on the percentage protein content of oyster tissue. Partitioning the diet effect showed a significant linear effect due to protein content in the diets ($F= 21.28$, $df=1, 15$, $P<0.001$) and no significant deviations from linear ($F=0.12$, $df=3, 15$, $P=0.95$). An ANOVA on the levels of dietary lipid showed no significant diet effect on percentage lipid composition of oysters.

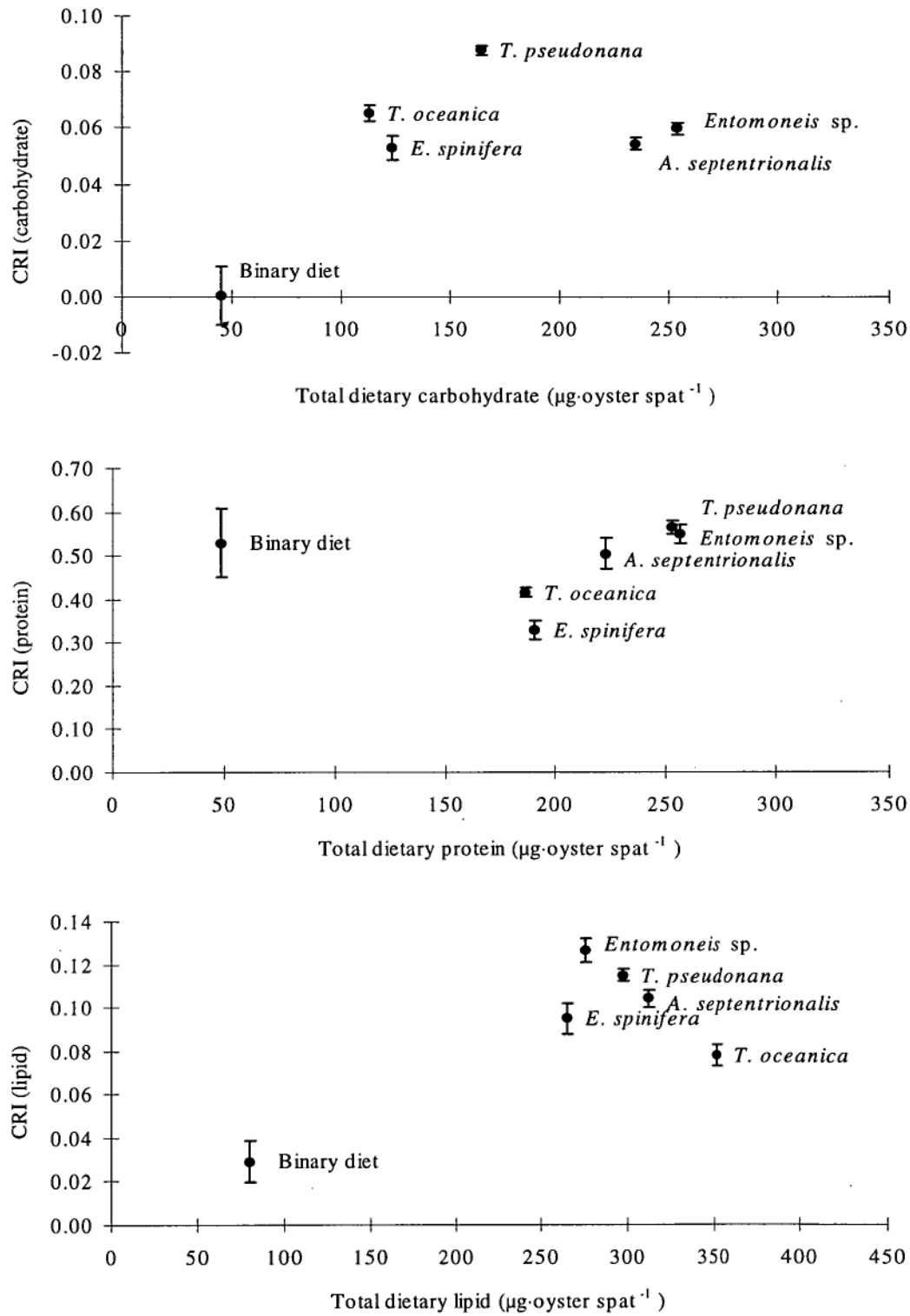


Figure 4.7 Oyster component retention indices (CRI) for carbohydrate, protein and lipid. Diets include the binary diet (*Isochrysis* sp. (T. iso) + *P. lutheri*) and the five ternary diets with the diatom component labelled. Error bars are ± 1 S.E. (n=4).

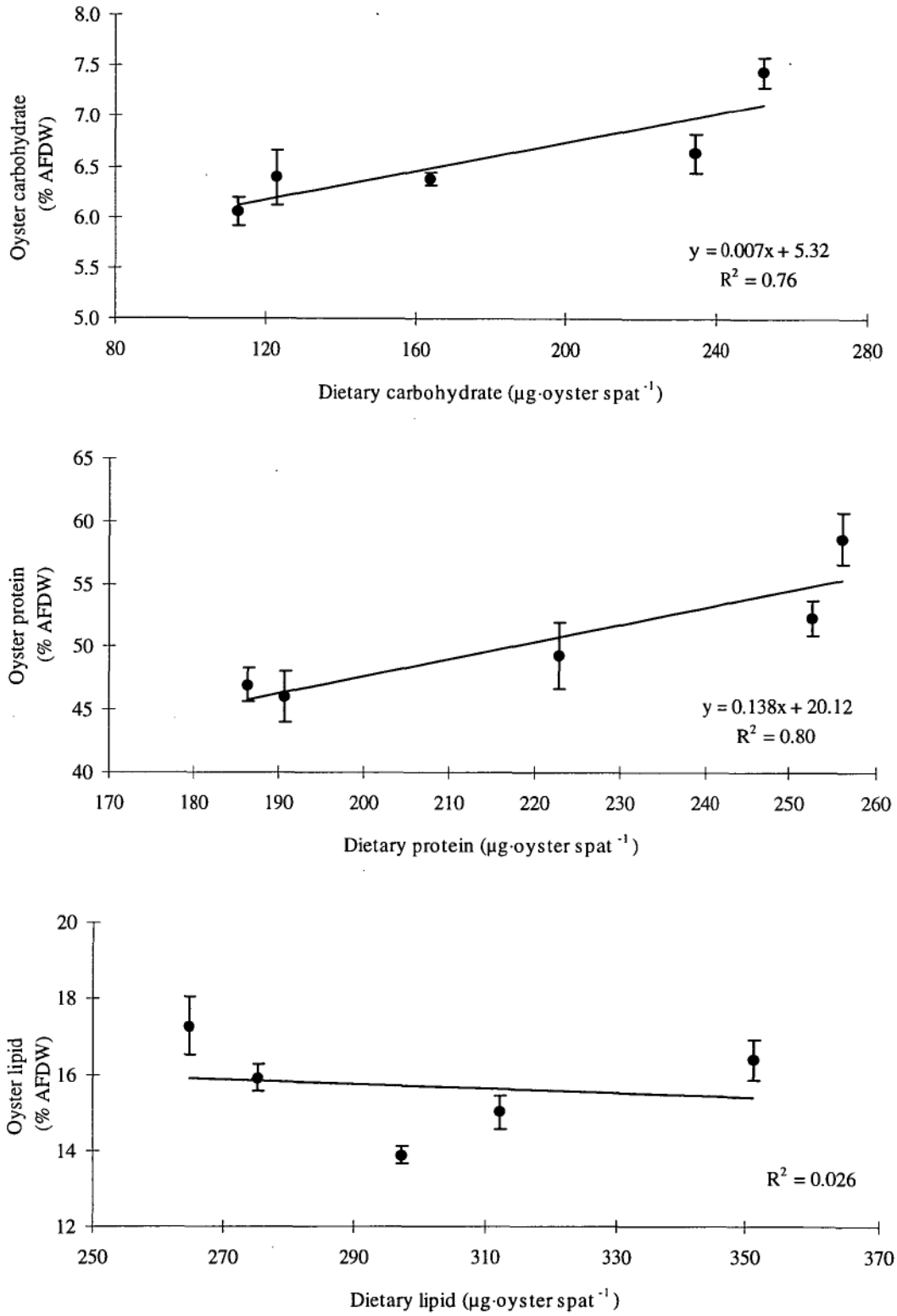


Figure 4.8 Relationship between ternary diets and oyster carbohydrate, protein and lipid. Error bars are ± S.E. (n=4).

4.4. *Discussion*

Two diatom isolates, *Entomoneis* cf. *punctulata* and *Attheya septentrionalis,* were shown to be excellent feed species for juvenile Pacific oysters. Each supported 90% of the growth rate of oysters fed the control diet containing *Thalassiosira pseudonana.*

Determining relationships between oyster growth and the gross composition of algal diets is complicated by diet-specific factors such as effects of trace nutrients, digestibility or limitation of a specific diet component. Oyster growth has often been explained in relation to the gross composition of the algal diet. However, these diets often include microalgae from a range of classes where there are not only large differences in gross composition but also where diet-specific factors can occur. In this study oysters were fed ternary algal diets, which reduces the likelihood of growth limitation caused by a deficiency in a trace nutrient. The algal class composition of the ternary diets was fixed which further reduces diet-specific factors such as differences in digestibility of algae. Therefore, the inclusion of a binary diet with one of five diatoms and the use of semi-continuously harvested algal cultures reduces diet-specific factors so that differences in oyster growth are more likely to be due to differences in the gross composition of the diets.

Growth rates for oysters fed the better performing diets (0.05-0.055·d^{-1}), were similar to other reported growth rates for mixed algal diets of between 0.06 to 0.08·d^{-1} (Enright *et al.*, 1986b; Thompson and Harrison, 1992). Stepwise regression analysis showed that protein was the single most significant dietary component in determining oyster growth. This was true when analysing just the five ternary diets or for all seven treatments. No other dietary component significantly affected oyster growth although total dietary energy was the best alternative variable. Oysters also retained much more of the dietary protein than either lipid or carbohydrate. A high component retention index (CRI) is expected if protein was not being catabolised as a primary source of energy. In *Ruditapes decussatus* seed, dietary protein had the highest retention index (0.6-0.88) which was inversely related to total dietary protein (Albentosa *et al.*, 1996). This was not found in this study where the CRI (protein) for the low-feed binary diet was similar

to that of the ternary diets. Also, the ternary diet containing the least protein had a significantly lower CRI (protein) than the binary and other ternary diets. However, like Albentosa *et al's* study, protein was also shown to be conserved and not readily catabolised during starvation. This was demonstrated by a high CRI (protein) in oysters fed the binary diet and by the near total conservation of protein in the unfed control oysters. Conservation of oyster protein in the unfed oysters indicates that the protein present in the initial oysters was performing essential functions and not available to be catabolised for energy. Increasing dietary protein levels resulted in increased percentage protein levels in the oyster tissue, primarily at the expense of tissue lipid. Over the experimental period, oysters in the nursery upweller from which initial oysters were taken grew to a similar level as those fed the binary diet, so nursery growth was not maximal. This is consistent with findings of McCausland *et al.* (1998) which showed improved growth through supplementary feeding of nursery oysters. The final protein composition of nursery oysters was unchanged from the initial (41.5% AFDW) and significantly lower than oysters fed the binary and ternary diets (46.0 - 58.6% AFDW). This is further evidence that protein was limiting the growth of initial oysters and why dietary protein correlates so strongly to oyster growth. Kreeger and Langdon (1993), found high O/N ratios in juvenile mussels from Yaquina Bay, Oregon indicated that growth of wild mussels was limited by dietary protein bioavailability. They suggest that a dietary protein level <40% w/w and a C/N >10, can limit the growth of juvenile *Mytilus trossulus*.

The amino acid profiles of a range of mariculture microalgae are very similar, irrespective of algal class (Brown, 1991). Algal amino acids have been found to closely match those found in oyster larvae (Brown *et al.*, 1997) and juvenile mussels (Kreeger and Langdon, 1993). Closely matched essential amino acid profiles between algae and consumers indicate protein of high nutritional value. In the ternary diet containing *Extubocellulus spinifera* the CRI (protein) was significantly lower than all other diets including the binary diet. Since protein from the binary diet was well assimilated and the CRI (carbohydrate & lipid) was similar to the other ternary diets this result indicates a low bioavailability of the *E. spinifera* protein and not a low ingestion of the diatom component of the diet. The ternary diet containing *E. spinifera* contained the least

protein and since protein is correlated to oyster growth, a lower growth is expected. However, the low bioavailability of protein from *E. spinifera* has resulted in a lower than predicted oyster growth.

Carbohydrate is reported to be the primary energy source for *Ostrea edulis* (Holland and Hannant, 1974). This was also found in this study with unfed oysters loosing 48% of their initial carbohydrate. The low retention index for carbohydrate in oysters fed all algal diets also indicates that most ingested carbohydrate is being metabolised for energy. The binary diet was deficient in carbohydrate as was evident by an oyster CRI (carbohydrate) of zero. This meant that the percentage carbohydrate content of the oyster flesh fell as the oysters grew. The nutritional condition of *Crassadoma gigantea* larvae has been correlated with the content of dietary carbohydrate (Whyte et al., 1990). As the dietary quantity of carbohydrate increased the percentage carbohydrate of the oyster flesh increased. This trend was evident not only for the equal-ration ternary diets, as it was for protein, but also for all treatments including the unfed control. It is likely that the oysters fed the high carbohydrate diets were in better nutritional condition even though overall growth was correlated to dietary protein.

Both carbohydrate and lipid are important energy reserves for juvenile oysters. In free swimming larvae, lipid has advantages as an energy reserve whereas glycogen reserves are more suited to adult oysters (Holland and Hannant, 1974). Lipid was an important source of energy for oysters fed all diets. The lipid content of the unfed oysters fell 37% during the 21 days of the experiment. The low retention index for lipid in oysters fed all algal diets also indicates the importance of lipid as an energy source. The CRI (lipid) in oysters fed the binary diet was significantly lower than those fed the ternary diets. This is likely the result of the binary diet being deficient in carbohydrate, which has put a greater demand on the dietary lipid to supply the oysters energy requirements. There was no correlation between the total dietary lipid and the percentage lipid in the oyster flesh. Increases in the percentage composition of carbohydrate and protein in the oyster flesh has been accommodated by a more variable lipid component.

Although the PUFAs 20:5n-3 and 22:6n-3 have been shown to be essential for oysters (Langdon and Waldock, 1981), the exact diet requirement is unknown. Growth of *C. gigas* larvae has been correlated to dietary levels of the energy rich short chain fatty acids 14:0 and 16:0 and even negatively correlated with the proportion of dietary PUFA 20:5n-3 (Thompson *et al.*, 1993). However, chlorophyte diets, deficient in essential PUFAs, adversely effects oyster growth (Langdon and Waldock, 1981; Brown *et al.*, 1989) yet essential fatty acids may normally be in excess in a mixed algal diet. Oysters fed the ternary diet containing *Entomoneis* cf. *punctulata* grew equal second to those fed the diet containing *T. pseudonana,* yet analysis of logarithmic and stationary phase cultures of this diatom showed it to contain no 22:6n-3. Therefore, the oysters must have obtained sufficient 22:6n-3 from the binary component of the diet since elongation and desaturation of n-3 fatty acids is reported to be insufficient to support active growth (Waldock and Holland, 1984). The prymnesiophytes in the binary diet; *Isochrysis* sp. (T. iso) and *Pavlova lutheri* are both rich sources of 22:6n-3 containing 8.3 and 9.4 $g \cdot 100g^{-1}$ (of total fatty acid fraction) respectively (Volkman *et al.*, 1989). Assuming an average 22:6n-3 content of 9 $g \cdot 100g^{-1}$ of total fatty acid and that fatty acids constitute 20-40% of the total lipid (Cohen, 1986) then the binary diet may be expected to have contributed a total of 0.4-0.8 mg of 22:6n-3 to the ternary diets. This equates to 0.07-0.14 μg 22:6n-3$\cdot$oyster $^{-1}\cdot$day $^{-1}$ and means that the oysters fed the ternary diet containing the *Entomoneis* cf. *punctulata* were not limited at this dosage. Thompson *et al.* (1993) suggests that there may be threshold values for the oysters requirement for PUFAs. For 20:5n-3 this threshold may be as low as ~1.3% of total fatty acids and for 22:6n-3 there may be a beneficial range of 0.5 to ~2%. Whyte *et al.*, (1990) found that a dietary 22:6n-3 level of 4% of fatty acids, appeared sufficient to maintain a level in *Crassadoma gigantea* larvae of 8.9%

4.5. Conclusions

Attheya septentrionalis and *Entomoneis* cf. *punctulata,* were shown to be excellent feed species for juvenile Pacific oysters. The deficiency in the essential PUFA 22:6n-3 in *Entomoneis* cf. *punctulata* was compensated for when it was used as part of a mixed diet with the prymnesiophytes *Isochrysis* sp. (T. iso) and *Pavlova lutheri.* The protein

content of each diet was the most significant factor in determining oyster growth with high protein diets supporting faster growth. The reduced performance of the two test diatoms, *T. oceanica* and *E. spinifera* appeared to be due to too little protein. For *T. oceanica,* protein was reduced due to its high lipid content while in *E. spinifera* protein levels were low and apparently less bioavailable than in the other species. The composition of the diet effected the final composition of the juvenile oyster tissue. High levels of dietary protein and carbohydrate resulted in oyster tissue with higher percentage compositions of these fractions at the expense of tissue lipid.

Test diatoms were selected because of favourable growth characteristics and a gross composition rich in carbohydrate or lipid. The results have shown that for the selected juvenile oysters a high protein diet would be more beneficial. *Nitzschia* cf. *paleacea* (CS-429) and *Minidiscus trioculatus* (CS-435) are two protein-rich isolates that have reasonable growth characteristics (Table 3.2) and accumulate protein as they enter stationary phase (Table 3.3). It is possible that these two species would also make excellent feed species for juvenile molluscs.

Chapter Five

Production of Algal Concentrates by Centrifugation

Chapter 5

Production of Algal Concentrates by Centrifugation

5.1 *Introduction*

In a survey of bivalve hatcheries and nurseries, Coutteau and Sorgeloos (1992) found that more than 50% of operators claimed to have experimented with artificial diets. This showed the high level of interest by commercial bivalve operators for use of alternatives to live algal cultures. Such diets could be used to boost production or to save on the high cost of maintaining a live algal culture facility. However, there is little published data by commercial operators on the effectiveness of the tested algal diets for replacing or partially substituting the demand for live algae. Coast Oyster Company (Quilcene, U.S.A.), produce algal concentrates with the intent of feeding remote set larvae (Donaldson, 1991). They report that it is their experience that live algae are always a better food source than any of the algal substitutes that they have tested.

Innovative Aquaculture Products Ltd. undertook a survey into various aspects of centrifuging marine microalgae for use in its oyster hatchery (Watson *et al.*, 1986). They found that *Thalassiosira pseudonana* and *Chaetoceros calcitrans* withstood the centrifuge process well and the cells were easily resuspended. The prymnesiophytes, *Isochrysis galbana* and *Pavlova lutheri* were prone to severe damage. When the concentrated algae (paste) were stored at 4°C, food value declined with time and pastes had a typical shelf life of 2-3 weeks. Donaldson (1991) also found that pastes stored in this way had a shelf life of only 10 days.

In one of the few published experiments evaluating stored algal concentrates, Nell and O'Connor (1991) found that a mixed algal paste diet of *P. lutheri* and *C. calcitrans* produced a greater growth of *Saccostrea commercialis* than any other fresh or stored, single or combined diet tested. Centrifugation and storage of algae had varied effects on different species, but it had no significant deleterious effects on any of the diatoms.

This chapter will investigate the effect of centrifugation on microalgae with the aim of producing data on the nutritional value of algal pastes as bivalve feeds. Research will focus on diatom species that are reported to show most potential for producing a quality algal paste. In the chapter, I discuss methods to produce algal concentrates by centrifugation, and to assess their nutritional value for juvenile Pacific oysters.

5.2 Materials and methods

5.2 i. Microalgal cultures

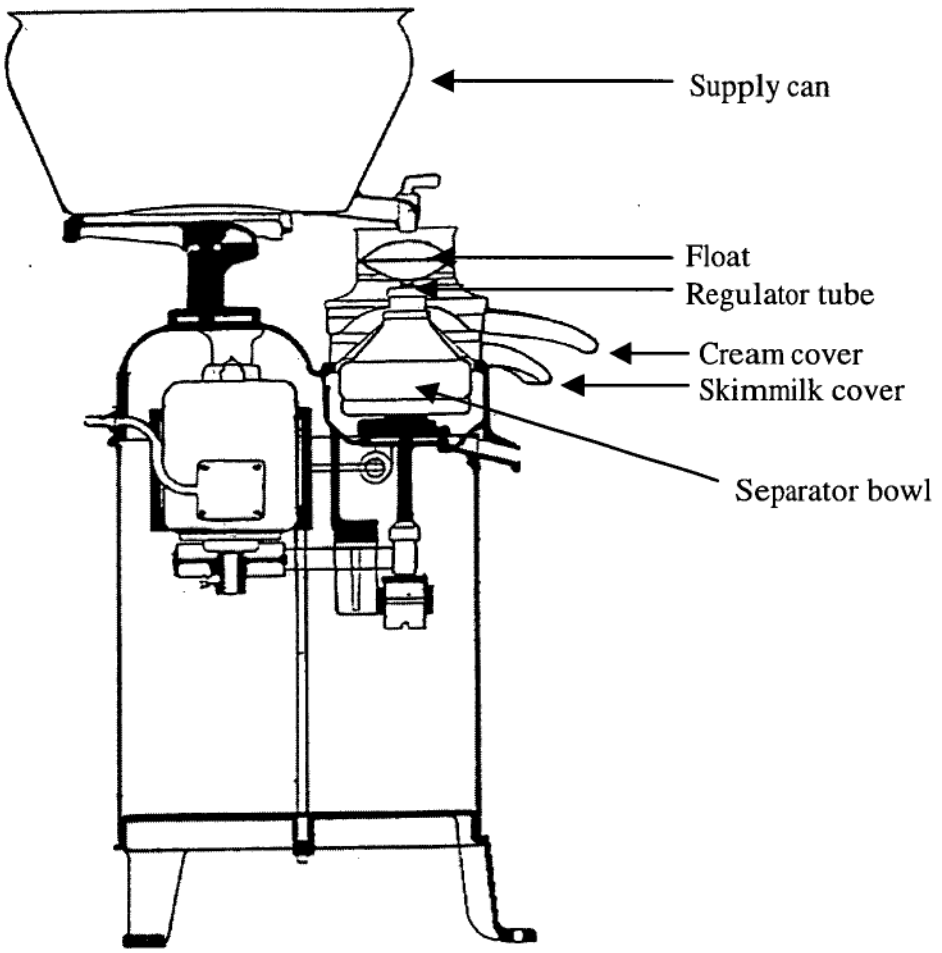
Microalgae from six algal classes were assessed for their ability to withstand centrifugation during the production of algal concentrates. Culture volumes of 10 L (carboys) to 500 L were used in assessment trials. Carboy cultures were grown as outlined in section 2.1 i. Larger volume cultures were grown in polyethylene bags, a polycarbonate tank or open polyethylene tubs. Most algae species used for centrifugation trials were grown in polyethylene bags (80 L). However, diatoms failed to grow well or predicably in this system so were cultured in the large polycarbonate tank (140 L) or polyethylene tubs. Conditions for these cultures were similar to the carboy cultures, but were less controlled than those in environmentally controlled growth cabinets. Temperature was partially regulated by airconditioning in the range 18 ± 5°C and illumination was by cool white fluorescent lights, either continuously or 12:12 h light/dark cycle. Tub cultures (500 L) were illuminated by mercury vapour lamps, supplemented with natural daylight. Modified f/2 media (Table 2.1) was used for all cultures. Aseptic techniques were used during initiation and maintenance of large-scale cultures, but all were non-axenic when harvested.

5.2 ii. Paste production

Algal pastes were produced by centrifuging cultures using a modified Alfa Laval cream separator type 106AE. The centrifuge is designed to operate as a liquid/liquid separator producing cream and skim milk from whole milk. To collect algae the centrifuge was modified to operate as a liquid/solid separator (Figure 5.1 and 5.2). Culture inflow was controlled by replacing the supply can and float with a variable speed peristaltic pump with a feed line connected directly to the regulating tube. Liquid effluent was restricted to leaving via the cream cover by inserting a rubber o-ring on the top disc inside the centrifuge bowl. Solids (algal cells) were collected against the internal surface of the bowl hood. Production of algal pastes was a combination of four process streams; incoming culture, outgoing effluent, collected algal paste and a small (450 mL) liquid concentrate waste stream (Figure 5.3).

Each harvest cycle was kept at less than 120 min to minimise the period of time that algal cells were subjected to high gravitational forces. Cultures were harvested at 1-1.5 $L \cdot min^{-1}$ to achieve a harvest efficiency of ≥80% for a range of microalgae (see Figure 5.4). Efficiency was determined from the difference in chlorophyll or algal cell concentration between the incoming culture and the waste effluent stream. At completion of a harvest cycle the centrifuge bowl was removed and inverted to drain residual liquid (liquid concentrate). The bowls locking nut was removed in a sterile hood or laminar-flow cabinet, and the bowl hood carefully lifted off. Using aseptic techniques the algal paste was then gently scraped off from the bowl hoods internal surface (Figure 5.2 b) and placed in a sterile beaker kept on ice. The collected paste was then gently mixed with a spatula and transferred to pre-weighed sterile syringes, these were re-weighed, sealed with parafilm and refrigerated at 4°C.

A)



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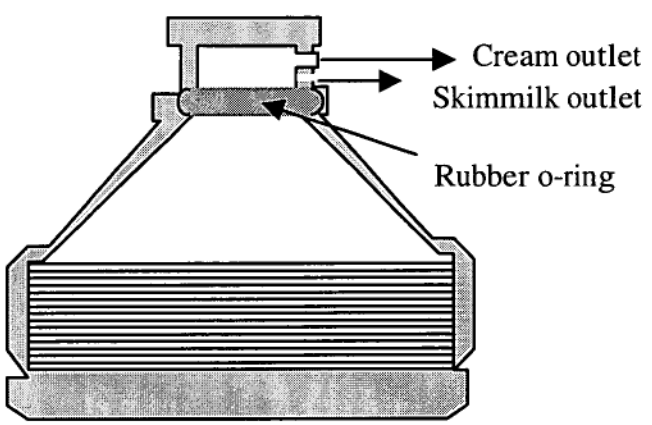
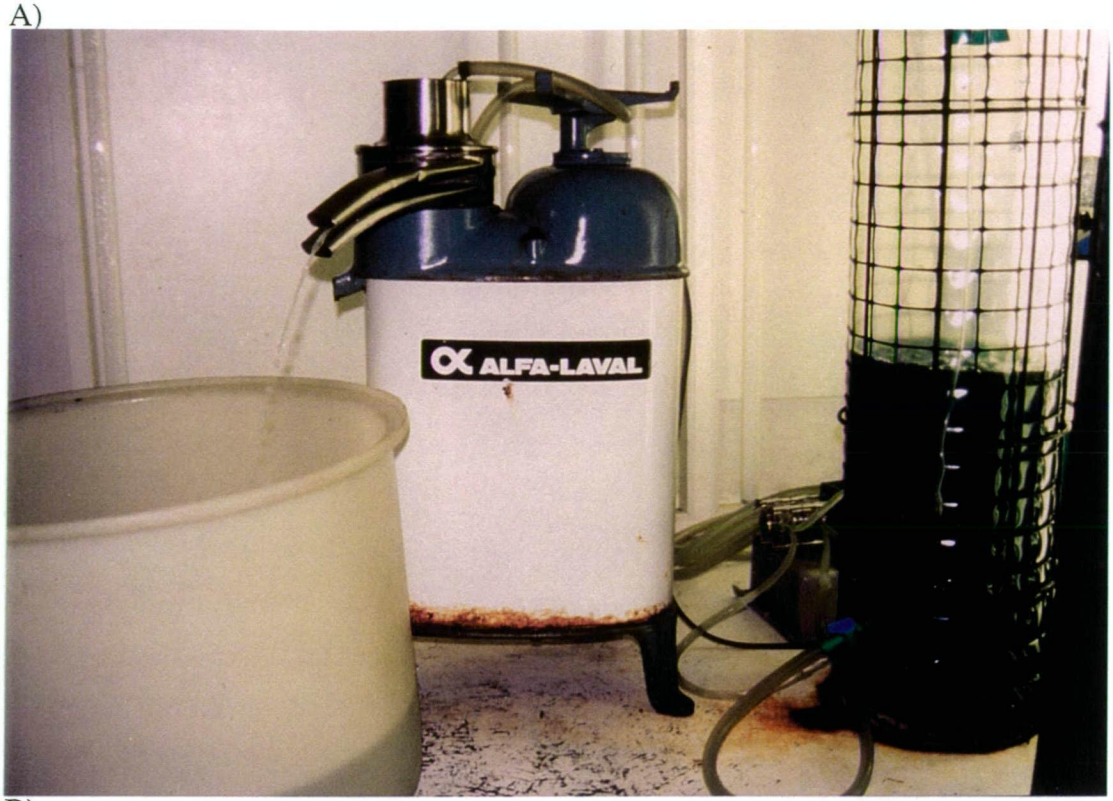


Figure 5.1 A) Cross-sectional view of Alfa Laval (106 AE) cream separator. B) Cross-sectional view of separator bowl showing placement of rubber o-ring to restrict outflow to the cream outlet.

A)



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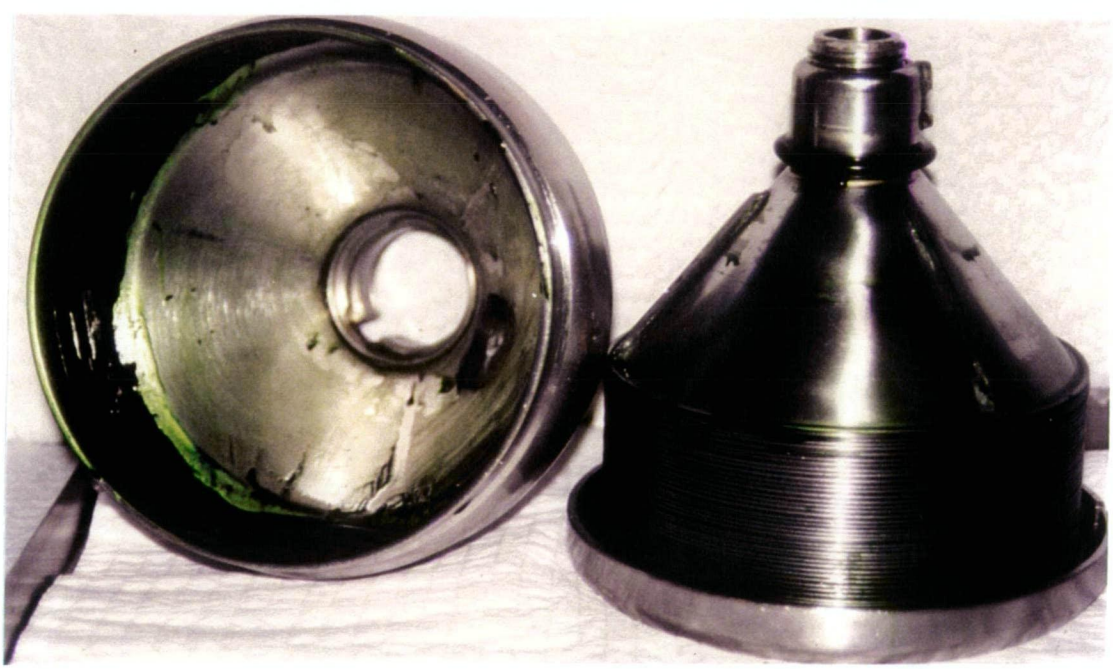
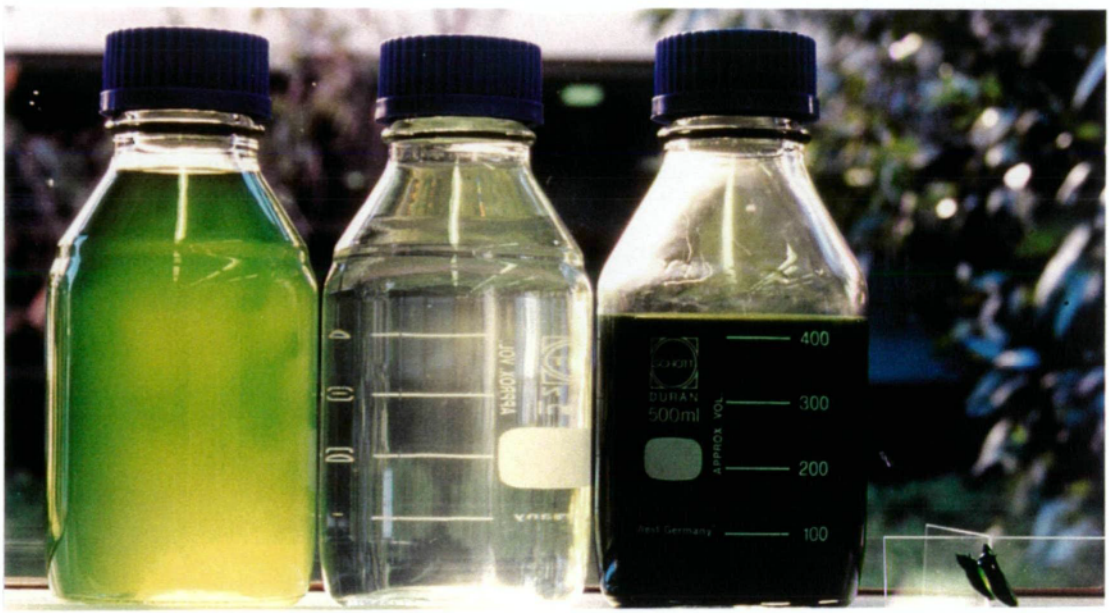


Figure 5.2 A) Centrifuge in operation harvesting an 80 L *Tetraselmis suecica* culture. A peristaltic pump is pumping the culture (feed stream) into the regulating tube and the clear waste (effluent stream) is leaving via the cream cover outlet.
B) Disassembled separator bowl showing the disk-stack with inserted o-ring and algal cells (pastes stream) collected against the internal surface of the bowl hood.



Centrifuge feed | Centrifuge effluent | Liquid concentrate | Algal paste

Tetraselmis suecica

Figure 5.3 Samples of the four process streams of algal paste production; feed, effluent, liquid algal concentrate and algal pastes. The feed stream was *Tetraselmis suecica* and the liquid cell concentrate is the total volume of this stream.

5.2 iii. Characterisation and evaluation of algal pastes

Subsamples of algal pastes were taken during storage periods for characterisation the pastes. Samples were either analysed immediately or stored for later analysis in liquid-N_2 or at -20°C.

The moisture content of the pastes was measured by weighing a sample of paste onto pre-weighed foil and drying overnight at 100°C. After re-weighing, the samples were combusted overnight at 450°C to calculate total AFDW. Samples were also resuspended and washed in ammonium formate (0.5M) to measure DW and washed AFDW (section 2.1 i.). The difference in the total AFDW and the washed AFDW was taken as the degree of cell leakage that occurs on resuspending pasted cells.

Analysis on samples of pastes included some or all of those shown in Table 5.1.

Table 5.1 Range of analysis performed on algal pastes and section number describing method.

Analysis	Sample storage (if required)	Method
Viable bacteria	Not stored	Section 2.1 iii.
Chlorophyll *a*	-20°C	Section 2.2 i.
Ascorbic acid	Liquid-N_2	Section 2.2 ii.
Protein, Carbohydrate and Lipid	-20°C	Section 2.2 iii.

Damage caused to algal cells from the pasting process was visually assessed by light microscopy of resuspended pasted cells (section 2.3 i.). Viability was assessed using Evans' Blue dye exclusion test (Withers, 1985). A stain solution of Evans' Blue, 0.025% (w/v of seawater), was prepared and one drop mixed with one drop of cell suspension on a microscope slide. A cover slip was applied and after 5 min the sample was washed twice with seawater and examined on a light microscope. Living cells remained unstained while dead cells accumulated the blue stain.

5.2 iv. Evaluation of Algal Pastes as Diets for Juvenile Pacific Oysters

5.2 iva. Feeding Experiment 5.1. Nutritional Value vs Storage Time

Algal paste diets of *Thalassiosira pseudonana* were evaluated against live algal diets in two feeding experiments with juvenile Pacific oysters using an experimental system outlined in section 2.1 ii. In the first experiment the nutritional value of pastes stored at 4°C for varying periods was evaluated. Freshly graded juvenile oysters (700 µm; 0.5 mL packed volume) were dispensed into each of 25 experimental upwellers. Five replicates of each treatment shown in Table 5.2 were run.

Table 5.2 Details of diets used in feeding experiment 5.1.

Treatment	Treatment description	
1	Unfed control	
2	Live *T. pseudonana* control diet	
3	*T. pseudonana* paste diet	0.5-2.5 weeks storage at 4°C
4	*T. pseudonana* paste diet	2.5-5.0 weeks storage at 4°C
5	*T. pseudonana* paste diet	11-13.5 weeks storage at 4°C

The live algal diet was grown in 10 L polycarbonate carboys (section 2.1 i.). Pastes were prepared from 500 L polyethylene tub cultures grown at Shellfish Culture's hatchery at Bicheno, Tasmania, Australia. To obtain pastes of different ages, 3 separate pastes were made in the 3 months prior to the start of the feeding experiment (Pastes 1-3; Table 5.3). To maintain the age of pastes fed to oysters, within the pre-set ranges (Table 5.2), an additional paste (Paste 4, Table 5.3) was made during the feeding experiment and the four pastes were fed as shown in Table 5.3.

Table 5.3 Details of the four algal pastes of *Thalassiosira pseudonana* used to formulate the 3 algal paste diets shown in Table 5.2. The age of each paste while it was used a feed is shown in brackets.

Diet	Feeding days 1-12	days 13-17
0.5 - 2.5 week old paste	Paste 3 (6-17 d)	Paste 4 (4-8 d)
2.5 - 5.0 week old paste	Paste 2 (22-33 d)	Paste 3 (18-22 d)
11 -13.5 week old paste	Paste 1 (78-89 d)	Paste 1 (90-94 d)

A 2 d pre-trial was undertaken to determine daily feed rate. Four buckets containing 0.5 mL of oysters were fed live *Thalassiosira pseudonana* at four feed rates (7.25, 14.5, 29 and 58 mg DW·upweller^{-1}). After 24 h feeding, consumption was measured by the change in chlorophyll *a* levels and converted to DW values from the initial culture chlorophyll *a* and DW measurements. There was a maximal feeding rate of 5.5 mg DW·upweller^{-1} which was met at all feed rates except the lowest rate (7.25 mg DW·upweller^{-1}). A daily feed rate of 14.5 mg DW·upweller^{-1} was the minimum feed rate that resulted in maximal consumption and this rate was set for the experiment. The DW of each algal paste was measured at the start of its use in the experiment; for the live algal culture it was measured daily. The feeding experiment ran for 18 d after which time the oysters were collected and DW and AFDW measured (section 2.1 ii.). Diets were assessed (section 2.4.) by comparing the instantaneous growth rate k (section 2.1 ii.), of the paste fed oysters to that of the live algal fed oysters.

5.2 ivb. Feeding Experiment 5.2. Blended Paste:Live Algal Diets

The second feeding experiment investigated the ability of blended diets of algal paste with a live algal component to compensate for potential nutritional deficiencies found in total paste diets. Freshly graded (900-1100 μm) juvenile oysters were dispensed (1.0 mL packed volume) into each of 25 experimental upwellers. Five replicates of each treatment shown in Table 5.4 were run.

Table 5.4 Treatment description and formulation of diets used in feeding experiment 5.2.

Treatment	Treatment description	Formulation
1	Unfed control	
2	Live *T. pseudonana* algal diet	100% live algae
3	*T. pseudonana* paste (0.5 - 2 weeks old)	100% paste
4	*T. pseudonana* blended diet	80:20 paste : live algae
5	*T. pseudonana* blended diet	60:40 paste : live algae

The live algal diet was grown in 10 L polycarbonate carboys (section 2.1 i.). Paste was prepared from 6 x 20 L glass carboys cultures grown at Shellfish Culture's nursery facility at Pipe Clay Lagoon. To maintain pastes of 0.5 - 2 weeks old, two algal pastes were prepared and used during the trial. All diets were added once a day at a rate of 10 mg DW·upweller^{-1}. The DW of each algal paste was measured at the start of its use in the experiment; for the live algal culture it was measured daily. The feeding experiment ran for 21 d after which time the oysters were collected and 4 x 50 oyster samples from each upweller were removed and stored for analysis (section 2.2 iii.). Oyster protein and carbohydrate were analysed from two of the samples of 50 oysters. For lipid, a 50 oyster sample from each replicate was combined (5 x 50 oysters) and the combined sample extracted. The last 50 oyster sample was stored in Liquid-N_2 as a backup set of samples. The remaining oysters (initial 1 mL minus 200 oysters) were analysed for DW and AFDW (section 2.1 ii.). Diets were assessed (section 2.4.) by comparing the instantaneous growth rate k, of the paste fed oysters to that of the 100% live algal fed oysters.

5.3 i. Algal Harvest Efficiency with Centrifugation

Centrifugation harvest efficiency for fourteen microalgae from six algal classes was examined (Table 5.5).

Table 5.5 Microalgae tested for production of pastes by centrifugation (Alfa Laval cream separator 106 AE).

Algal Class	Algal species	CSIRO code
Diatoms	*Attheya septentrionalis* (Østrup) Crawford	CS-425
	Chaetoceros calcitrans (Paulsen) Takano	CS-178
	Chaetoceros muelleri Lemmermann	CS-176
	Thalassiosira pseudonana (Hust.) Hasle & Heimdal	CS-173
Prymnesiophytes	*Isochrysis* sp. (T. iso)	CS-177
	Pavlova lutheri (Droop) Green	CS-182
	Pavlova pinguis Green	CS-375
Chlorophytes	*Stichococcus* sp.	CS-92
	Chlorella-like	CS-436
	Stichococcus-like	CS-437
Prasinophyte	*Tetraselmis suecica* (Kylin) Butcher	CS-187
Eustigmatophyte	*Nannochloropsis oculata* (Droop) Green	CS-179
Cryptophyte	*Geminigera cryophila* (Taylor & Lee) Hill	CS-138

The centrifuge has a separating capacity of 6 $L \cdot min^{-1}$, but for most microalgae the harvest efficiency fell below 70% at flow rates above 1.5 $L \cdot min^{-1}$. Figure 5.4 presents harvest recovery data taken after process stabilisation (15 min, Figure 5.5), for five algal species belonging to four classes. Different algae had different harvest efficiencies at set flow rates, but all decline with increasing harvest flow rate.

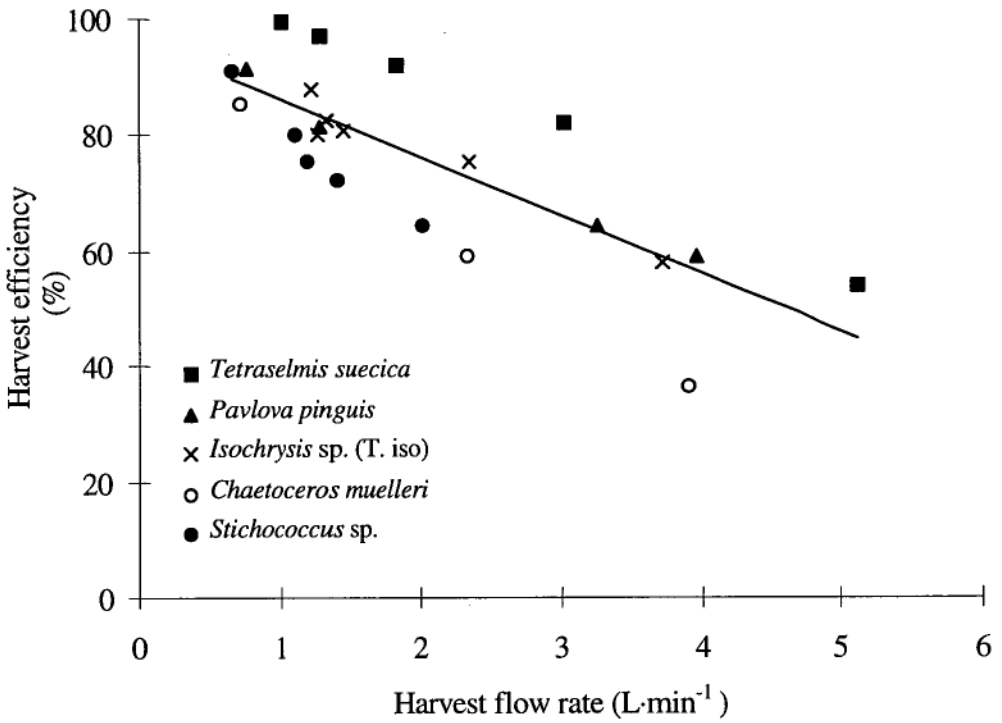


Figure 5.4 The harvest efficiency of five microalgae harvested using an Alfa Laval (106 AE) cream separator. Efficiency was measured from the difference in chlorophyll *a* concentration between the culture and effluent streams.

A regression line fitted to data of all five species, shows average harvest efficiency falls linearly according to the equation:

$$\text{Harvest efficiency (\%)} = -10.02 * \text{Harvest flow rate} + 96.30 \quad r^2 = 0.64$$

On average, a harvest rate of 1-1.5 L·min^{-1} gave a harvest efficiency of >70% for all microalgae. The prasinophyte *T. suecica*, was the most efficiently harvested microalgae with >95% harvested at a flow rate of 1 L·min^{-1}, falling to ~85% at 3 L·min^{-1}. The naked flagellate *G. cryophila*, had an apparent high harvest efficiency when measured by the difference in chlorophyll levels in the feed and effluent streams. However, it was the least efficiently recovered algae due to the total disruption of cells and the collection of only a gelatinous mixture of cell fragments. The small *Stichococcus*-like CS-437 (1 x 2 μm) was poorly recovered. This was because it failed to collect as a paste on the bowl hood, but only as a film on the surface of the bowl disks and was therefore not recoverable. Other small algae such as *Stichococcus* sp. were recoverable as a paste but

their harvest efficiency declined more rapidly with increasing harvest rate, falling from 90% at 0.65 $L \cdot min^{-1}$ to 65% at 2 $L \cdot min^{-1}$.

During the first 15-20 min of centrifugation, the harvest efficiency of the algal cells declined as the process stabilised. After this period, harvest efficiency declined only marginally over an extended period (120 min). The diatom *Chaetoceros calcitrans* was typical of other microalgae, its average harvest efficiency stabilising at 80-85% after 25 minutes of centrifugation (Figure 5.5). Harvest efficiency was related to centrifugation time according to the equation;

$$\text{Harvest efficiency} = -0.0001x^3 + 0.0209x^2 - 1.0726x + 99.75 \qquad r^2 = 0.99$$

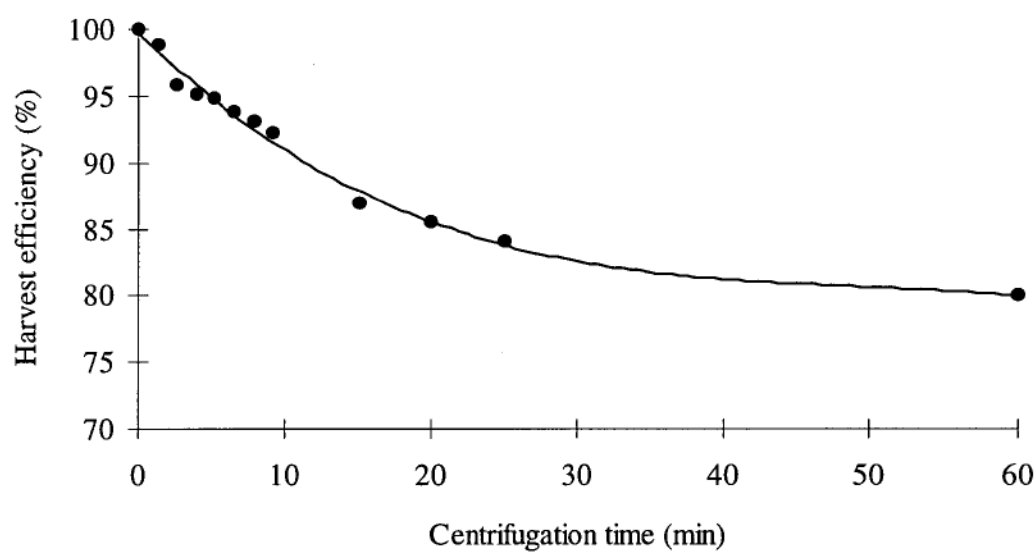


Figure 5.5 Algal harvest efficiency compared to centrifuge time for the diatom *Chaetoceros calcitrans*. Efficiency was measured from the difference in chlorophyll *a* levels in the culture feed and waste effluent streams at a flow rate of 1.1 L^{-1}.

During centrifugation the cells were concentrated over 2000-fold to produce a highly viscous paste. The moisture content of the pastes ranged between 65% and 84% (Table 5.6). Rigid diatoms produced pastes with the highest moisture content (82-84%). Centrifugation of more plastic species such as prymnesiophytes and smaller species

such as *Stichococcus* sp. gave pastes with a lower moisture content (72-77%). The largest alga, *Tetraselmis suecica*, was not only harvested most efficiently but produced a paste with the lowest moisture content 65%.

Table 5.6 Summary of cultures, harvesting and paste properties of a range of algal species harvested using an Alfa Laval cream separator (106AE).

Algal Class	Algal species	Culture	Culture harvest			Algal paste	
		Cell density ($cells \cdot mL^{-1}$)	Volume (L)	Rate ($L \cdot min^{-1}$)	Efficiency (%)	Paste recovery ($g \cdot 100\ L^{-1}$ culture)	Moisture (%)
Diatoms	*Chaetoceros calcitrans*	2.2×10^6	122	1.1	85	17.6	81.7
	Chaetoceros muelleri	1.1×10^6	70	1.2	84	22.4	83
	Thalassiosira pseudonana	2.2×10^6	195	1.4	77	13.4	84
Prymnesiophytes	*Isochrysis* sp. (T. iso)	3.6×10^6	127	1.1	83	15.8	72.3
	Pavlova pinguis	5.0×10^6	63	1	90	31.8	74.2
Chlorophytes	*Stichococcus* sp.	2.3×10^6	125	1	80	26.3	77.2
	CS-436 (*Chlorella*-like)	6.3×10^7	135	0.8	56	27.7	74.2
Prasinophyte	*Tetraselmis suecica*	5.8×10^5	132	1.2	98	18.9	65.4
Eustigmatophyte	*Nannochloropsis oculata*	2.3×10^7	213	1.1	79	18.8	74.3

5.3 ii. Cell Integrity after Centrifugation

With the exception of *G. cryophila*, all collected algal pastes were composed of whole cells. However, many species were damaged by centrifugation and leakage of cell components was evident (Figures 5.6 to 5.9). For small chlorophytes and *Nannochloropsis oculata,* light microscopy revealed little or no visible cell damage (Figure 5.9). Centrifugation and resuspension altered cells of Tetraselmis suecica (Figure 5.6). Their size increased slightly and their shape became ovoid with the loss of all flagella. However, cell integrity was maintained and there was no sign of cell leakage. Cells of *Thalassiosira pseudonana* and *Isochrysis* sp. (T. iso) were more damaged. Resuspended cells of *T. pseudonana* showed cells to have become more round and the cytoplasm to have retracted from the cell wall (Figure 5.8). This was even more evident in cells of *Isochrysis* sp. (T. iso) which also lost their flagella. *Isochrysis* sp. (T. iso) formed pastes that were 'rubbery' and semi-solid which did not readily resuspend, leaving most cells in clumps of up to several hundred (Figure 5.7).

Pastes of *T. pseudonana* (Pastes 1-4; Table 5.3) used as oyster diets in feeding experiment 5.1 had similar moisture (83.7%, CV= 0.7%), and total AFDW (10.50%, CV= 4.4%). After resuspending and washing cells (0.5M ammonium formate), the final AFDW values showed that there had been a considerable loss of organics from the pastes (Table 5.7).

Table 5.7 The percentage composition (moisture, DW, pre- and post-washed AFDW) and loss of AFDW for four pastes of *Thalassiosira pseudonana* used in feeding experiment 5.1.

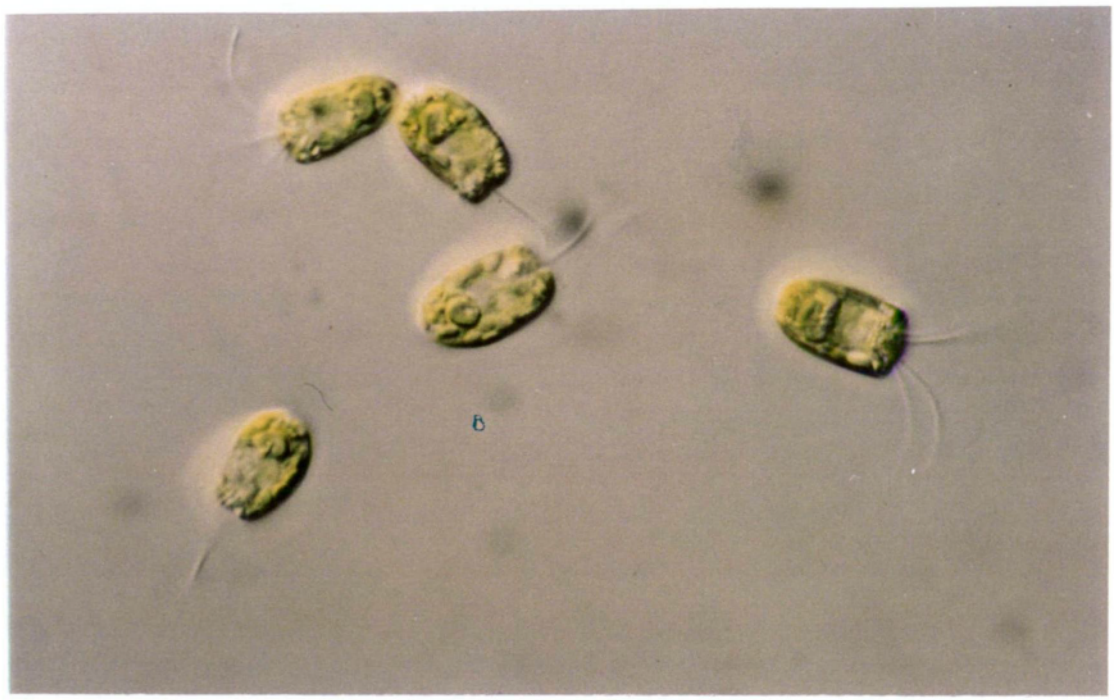
Paste	Paste age	Percentage composition of pastes				% loss of
			Pre-washed	Post-washed		
	(days)	Moisture	AFDW	DW	AFDW	AFDW
1	76	83.4	11.13	7.03	4.78	57
2	20	83.1	10.45	8.94	5.85	44
3	4	84.5	10.38	9.67	7.73	26
4	4	83.7	10.03	10.2	7.33	27

This loss was lowest in the youngest pastes (26% for 4 d old paste) and highest in the oldest paste (57% for 76 d old paste). Loss of organics (AFDW) in the pastes increased with time according to the logarithmic equation;

$$y = 10.49*Ln(x) + 11.93 \qquad r^2 = 1.0$$

where y= loss of AFDW (% of total AFDW) and x= age of paste (days).

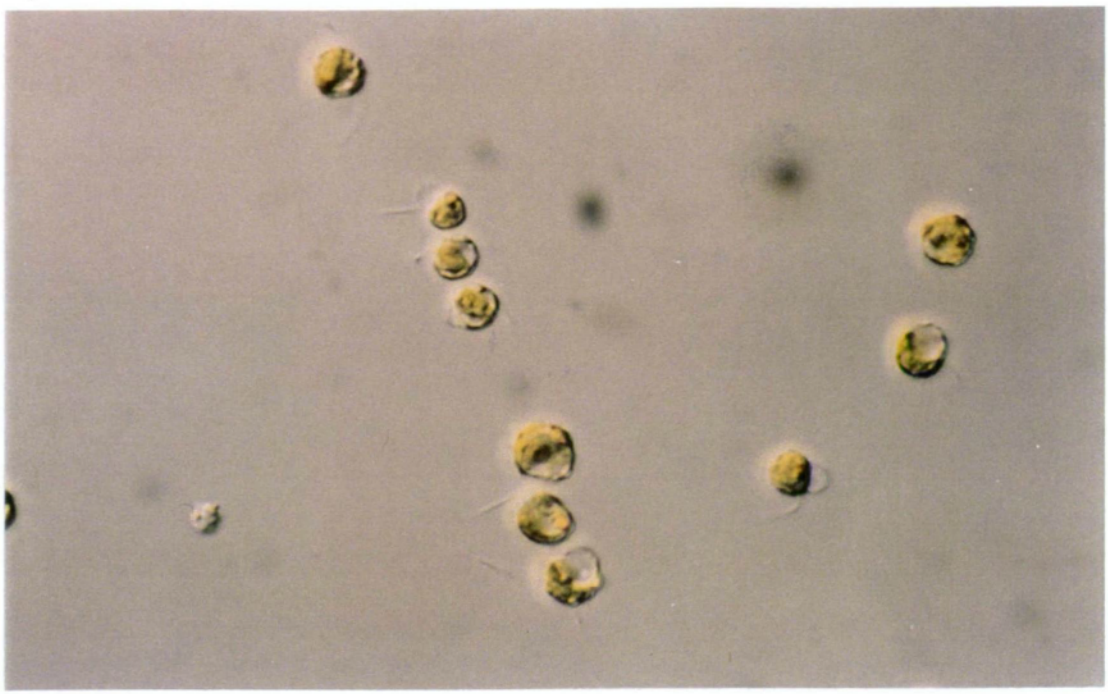
A)



B)

Figure 5.6 A) *Tetraselmis suecica* cells showing their characteristic shape and four flagella.
B) Resuspended algal paste of *Tetraselmis suecica* showing changes to cells resulting from centrifugation and resuspension. The cells are larger and the flagella are lost but the cells remain intact with the cytoplasm in contact with the outer membrane.

A)



B)

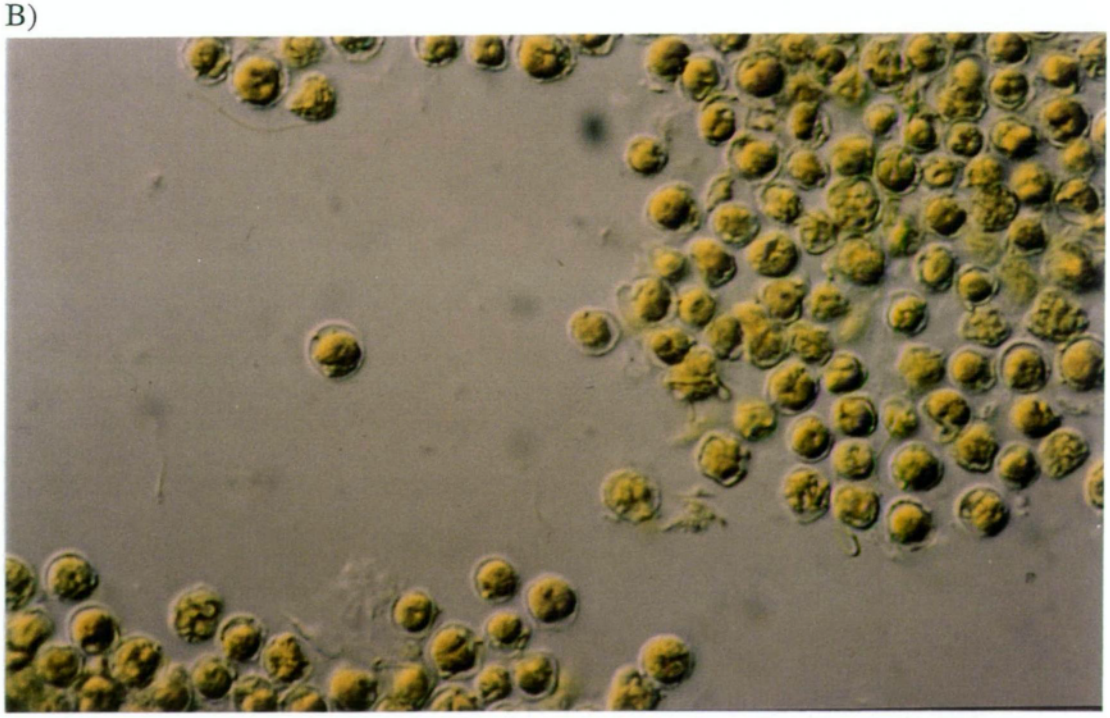
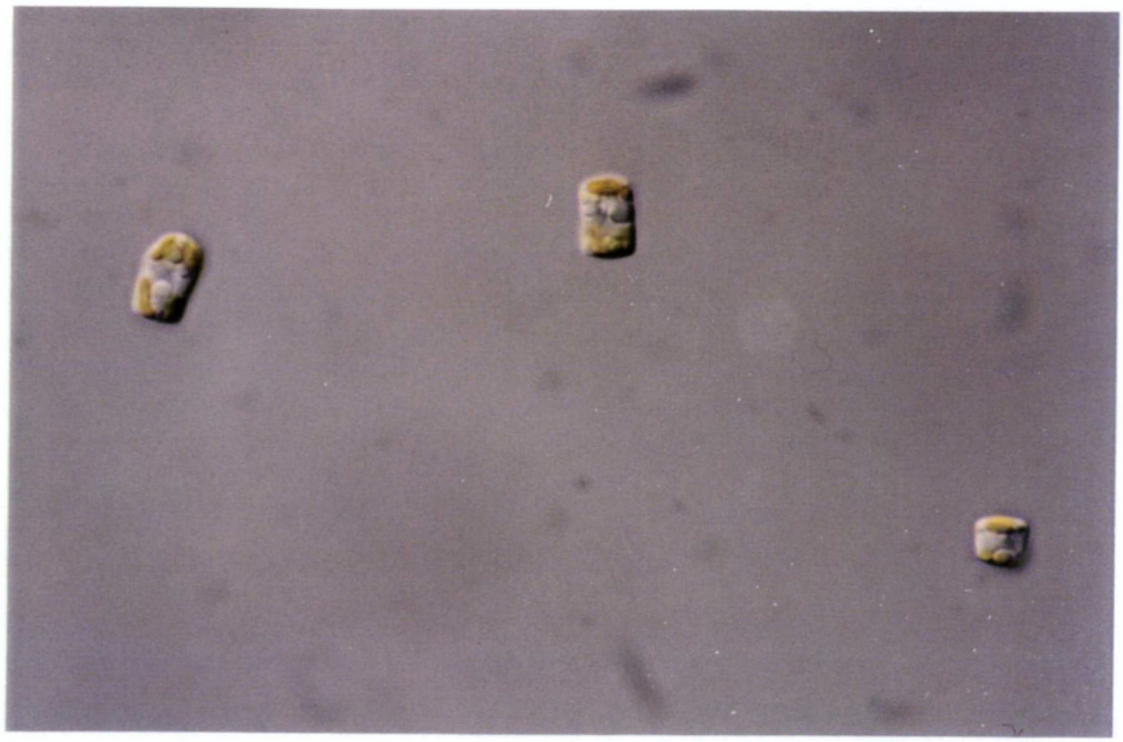


Figure 5.7 A) *Isochrysis* sp. (T. iso) cells with their two flagella visible on some cells.
B) Partially resuspended algal paste of *Isochrysis* sp. (T. iso) showing damage to cells resulting from centrifugation and resuspension. The flagella are lost and the cytoplasm has contracted from the outer membrane. The paste is gelatinous and cells do not readily dissociate, but remain as clumps of up to several hundred cells.

A)



B)

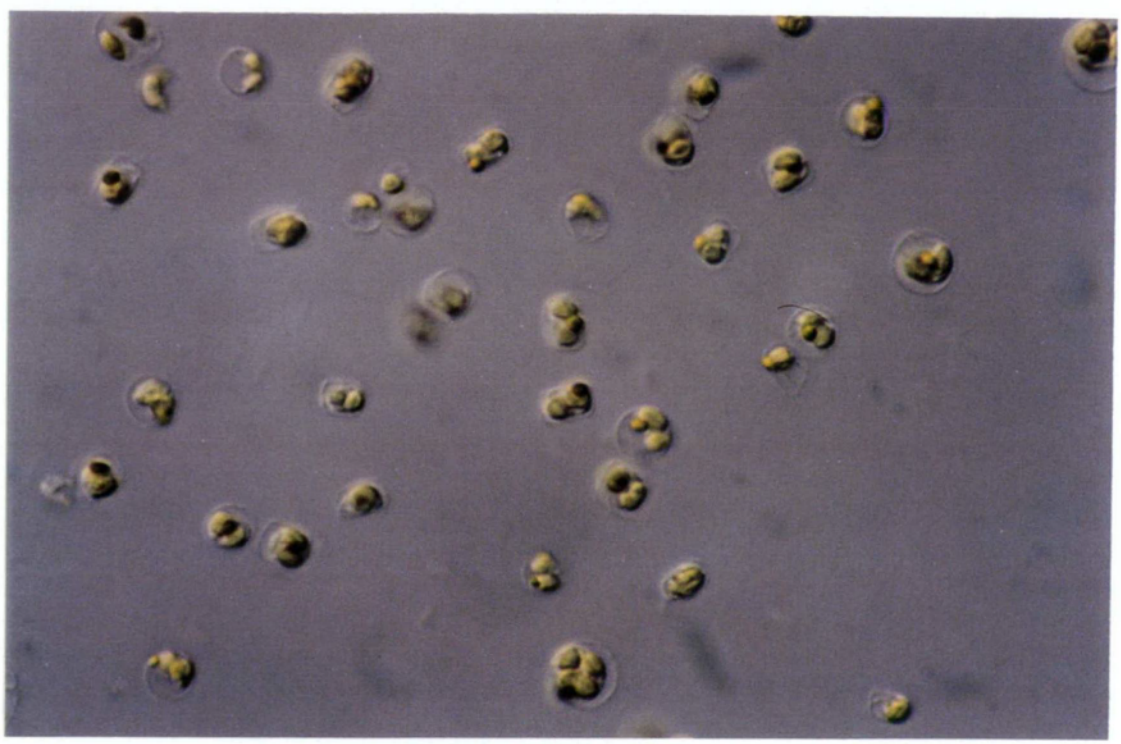
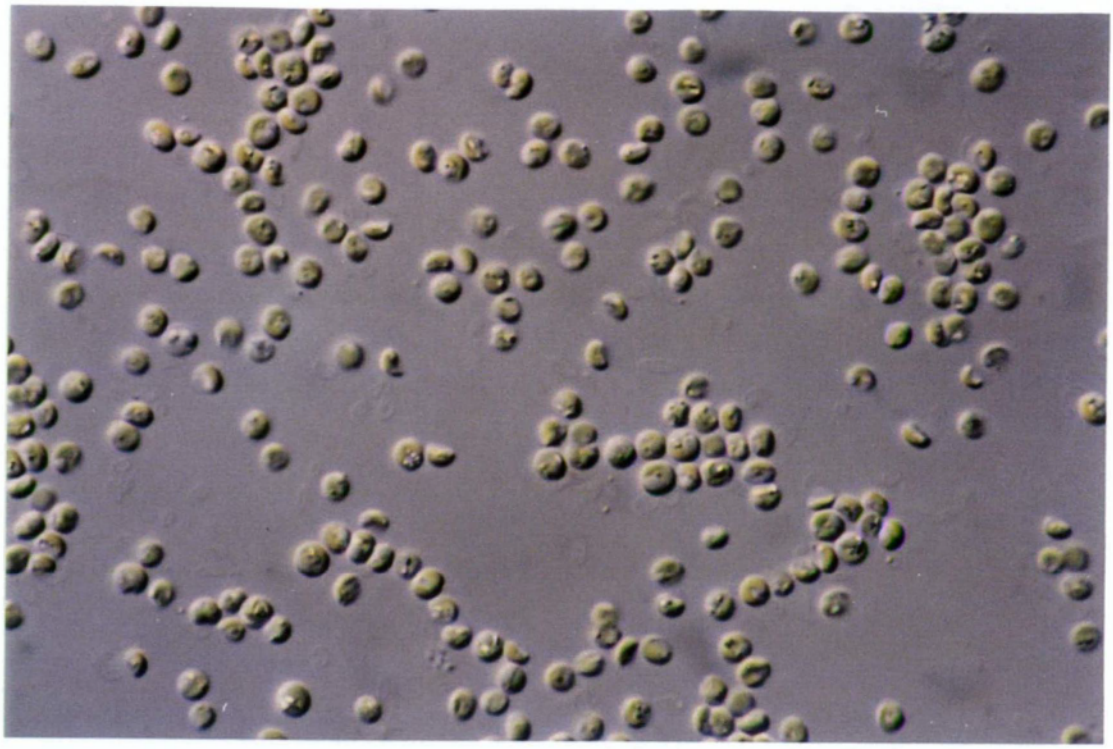


Figure 5.8 A) *Thalassiosira pseudonana* cells.
B) Resuspended algal paste of *Thalassiosira pseudonana* showing damage to cells resulting from centrifugation and resuspension. Many cells have become more circular with the cytoplasm contracting from the silica frustule.

A)



B)

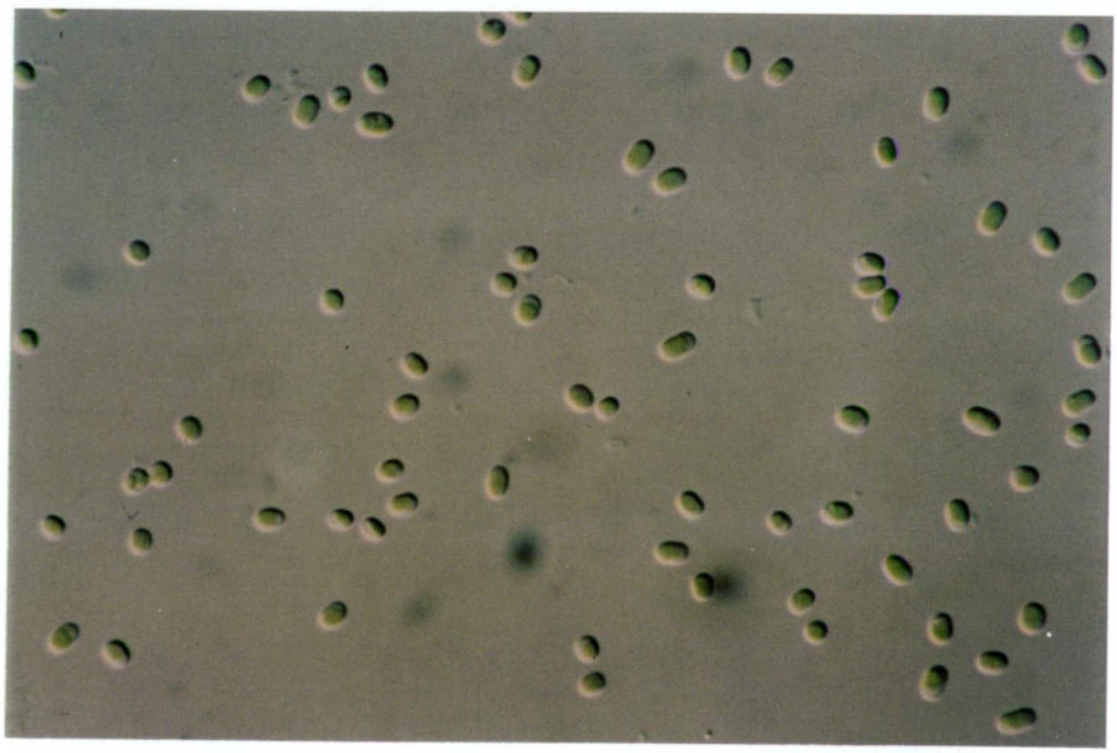


Figure 5.9 Two green algal species that show little discernible damage from centrifugation to form algal pastes are:
A) The eustigmatophyte, *Nannochloropsis oculata*.
B) The chlorophyte, *Stichococcus* sp.

5.3 ii. Chlorophyll Content and Stability

Analysis of the chlorophyll *a* content of pastes stored at 4°C, showed that there were two groupings of pastes. In one group were pastes of the green algae *Tetraselmis suecica, Nannochloropsis oculata* and the chlorophytes, *Stichococcus* sp. and isolate CS-436. In the other group were pastes of diatoms and prymnesiophytes.

For the green algal pastes, chlorophyll *a* levels remained relatively constant over extended periods of storage at 4°C. For one paste of *Stichococcus* sp., the chlorophyll *a* content increased from 3.3 to 5.5 $mg \cdot g^{-1}$ paste over the first 8 days (Figure 5.10).

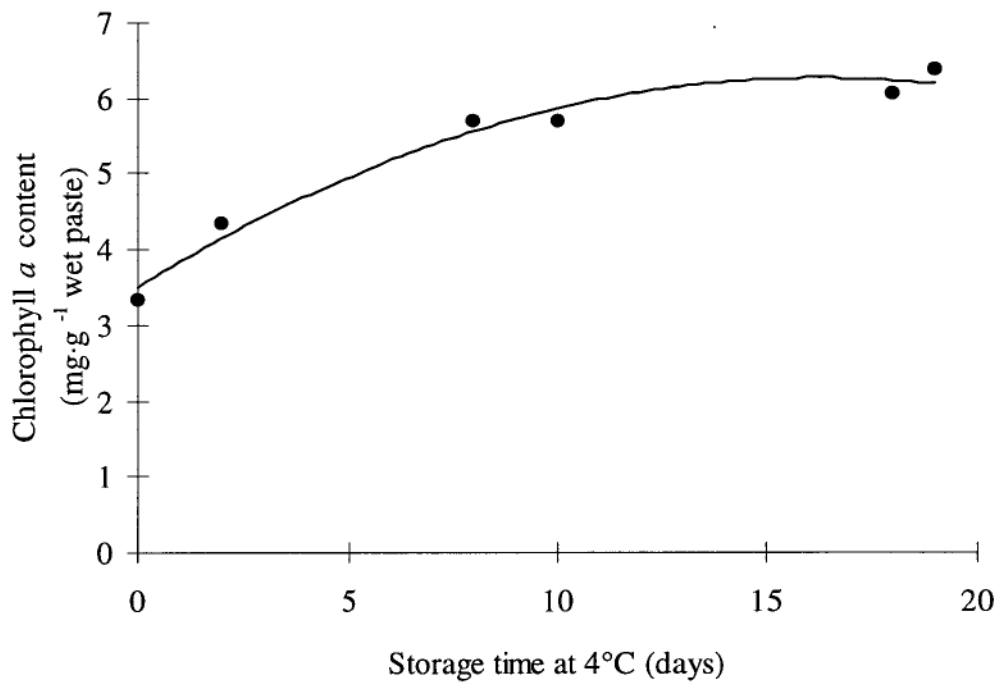


Figure 5.10 Chlorophyll *a* content of a paste of *Stichococcus* sp. During the first three weeks of storage at 4°C. $y = -0.010x^2 + 0.339x + 3.513$ $r^2 = 0.97$.

This trend was not observed in another paste of *Stichococcus* sp. that started with a chlorophyll *a* content similar to that reached by the first paste (Figure 5.11). In this paste the concentration remained almost constant until day 110, when it began to fall and scans indicated the presence of chlorophyll *a* breakdown products. For *T. suecica*, chlorophyll *a* levels had fallen only 10% after 257 days of storage and scans of the

extracts showed no indication of chlorophyll *a* breakdown products. For the chlorophyte CS-436, chlorophyll *a* concentrations fell <10% during 136 d storage, 4°C.

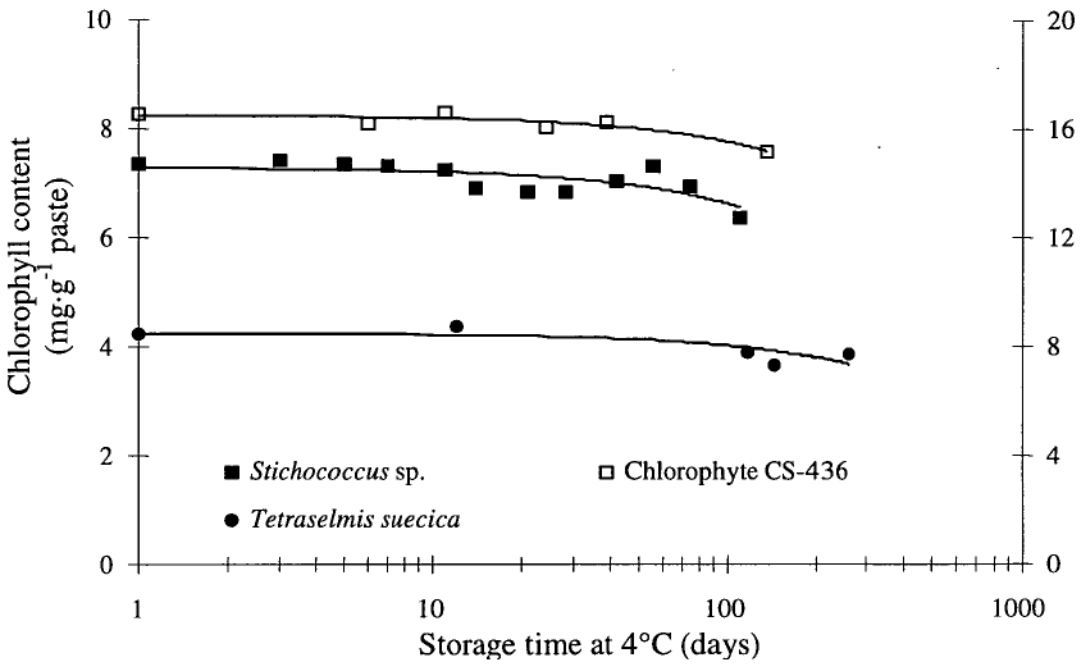


Figure 5.11 Chlorophyll content of pastes of three green algal species stored at 4°C for up to 257 days. The chlorophytes *Stichococcus* sp. And isolate CS-436, are represented on the y-axis. The prasinophyte *Tetraselmis suecica* is represented on the secondary y-axis. To separate data points, storage time is on a logarithmic scale.

Chlorophyll *a* concentrations in pastes from the second group of species fell over the first three weeks of storage. For pastes of *Thalassiosira pseudonana*, the average chlorophyll *a* concentration fell from 4.2 to 2.7 $mg \cdot g^{-1}$ paste over three weeks. Levels were then relatively stable (Figure 5.12). Scans of chlorophyll extracts of *T. pseudonana* paste taken after 0, 7, 10 and 24 days of storage showed that by day 7 the peak at λ_{max} 430 nm (Soret peak) was beginning to shift to λ 410 nm. By day 24 the shift was complete with the Soret peak having a λ_{max} 410 nm and no peak remaining at λ 430 nm (Figure 5.13a). This spectra is characteristic of the chlorophyll *a* breakdown phaeopigments, phaeophytin and phaeophorbide (Figure 5.13c). By day 10 other peaks associated with phaeopigments were also evident. These were a peak at λ 535 nm, loss of the peak at λ 580 nm and a shift in the peak at λ 616 nm to λ 609 nm. For pastes of

the chlorophyte CS-436, which had stable chlorophyll *a* concentrations, scans of chlorophyll *a* extracts of initial and day 136 samples revealed no shift in spectra peaks that would indicate breakdown products (Figure 5.13b).

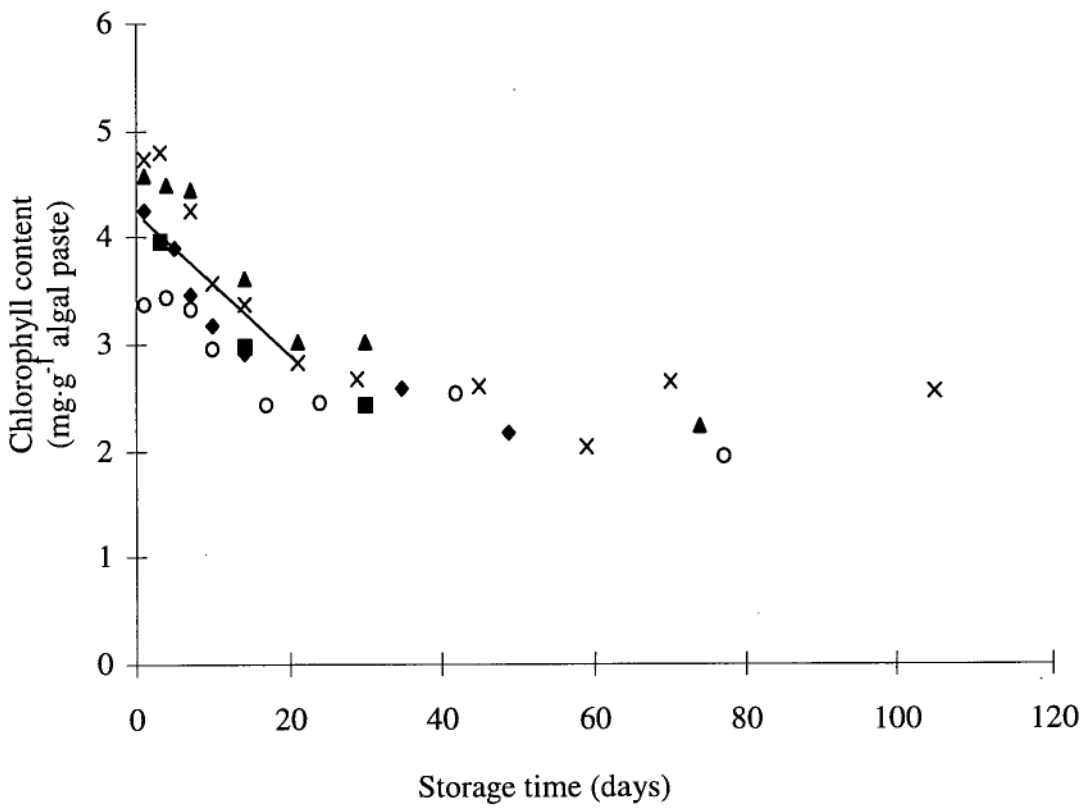


Figure 5.12 Chlorophyll concentration in five pastes of *Thalassiosira pseudonana* stored for extended periods at 4°C. Each paste is represented by a different symbol. All pastes were harvested from separate 500 L cultures and stored under identical conditions. Trend line is fitted to average values during the period of maximal change in chlorophyll levels ($y = -0.067x + 4.22$, $r^2 = 0.90$).

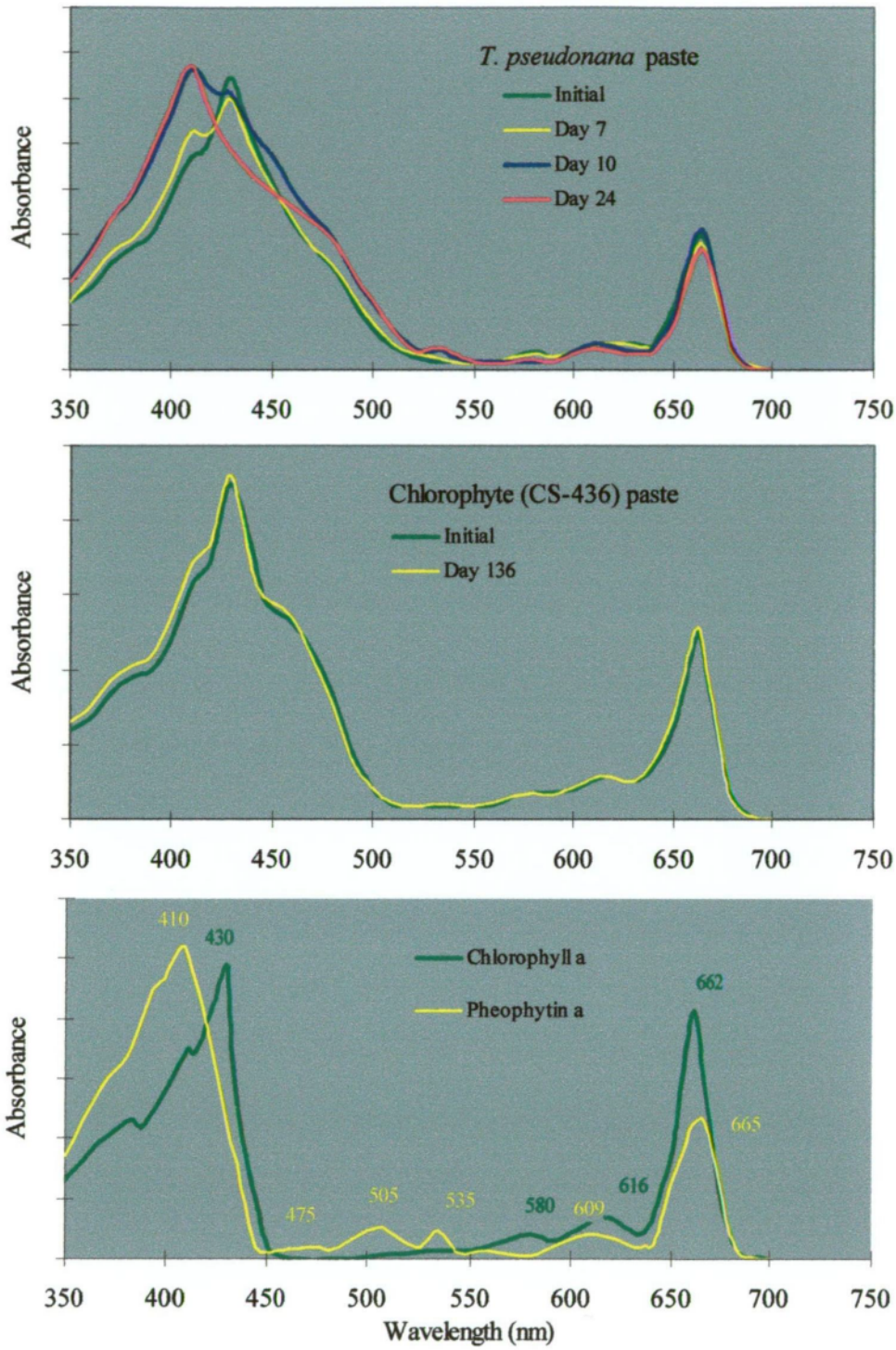


Figure 5.13 Scans of chlorophyll extracts (90 % acetone) of pastes of *Thalassiosira pseudonana* and isolate CS-246, stored for extended periods at 4°C. Scans are compared to those of purified chlorophyll *a* and its derivative phaeophytin *a* (100% acetone).

A) *Thalassiosira pseudonana*
B) Chlorophyte CS-246
C) Scans of purified chlorophyll *a* and phaeophytin *a* (reproduced from Jeffrey *et al.*, 1997).

5.3 iv. Bacterial Content of Algal Pastes

Mass cultures of all algal species were non-axenic when harvested, with bacterial numbers in the region 10^5 to $10^6 \cdot mL^{-1}$. Bacteria were concentrated with the algae during centrifugation. Because of their much smaller mass, they were not collected as efficiently with 25-50% leaving in the waste effluent stream. Approximately 10% were present in the liquid concentrate waste stream. On a mass balance 40-75% of the initial bacteria were expected to be in the paste. However, no more than 25% were routinely detected and a mass balance was not achieved.

In algal pastes the initial viable bacteria count was in the range of 10^7 to $10^9 \cdot g^{-1}$ paste. For most pastes stored at 4°C, numbers of viable bacteria fell 3 to 6 orders of magnitude within the first 10 days. After this period numbers began to gradually increase. Viable bacteria numbers in pastes of *Isochrysis* sp. (T. iso), *Pavlova pinguis* and *Chaetoceros muelleri* all fell at least 5 orders of magnitude within 10 days (Figure 5.14). For a *P. pinguis* paste the viable bacteria count fell from an initial level of $10^9 \cdot g^{-1}$ to $10^2 \cdot g^{-1}$ by day 10 and then increased to reach $10^5 \cdot g^{-1}$ by day 40. For pastes of *Thalassiosira pseudonana*, viable bacteria numbers followed a similar trend. However, the initial fall in numbers was seldom above 3 orders of magnitude and recovery in numbers was more rapid. Data from four pastes of *T. pseudonana* showed initial viable bacteria numbers falling from $10^9 \cdot g^{-1}$ to $10^6 \cdot g^{-1}$ by day 10 and then increasing to between 10^7 and $10^8 \cdot g^{-1}$ by day 35 (Figure 5.15).

In two algal pastes, levels of viable bacteria numbers did not drop but remained constant. This occurred in one paste each of *Chaetoceros calcitrans* and *Stichococcus* sp. stored at 4°C, where initial levels of 10^8 and $10^9 \cdot g^{-1}$ respectively, remained relatively constant over 40 and 20 days respectively (Figure 5.14). However, in a separate paste of each of these species the viable bacteria count followed a similar trend to *Isochrysis* sp. (T. iso).

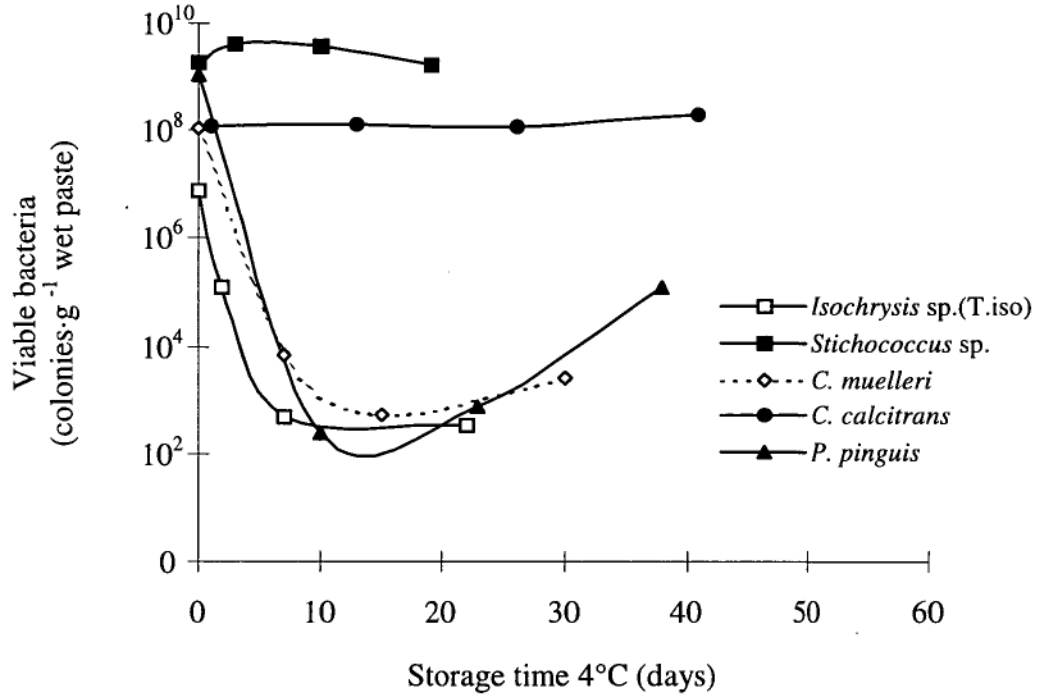


Figure 5.14 Levels of viable bacteria in algal pastes stored for extended periods at 4°C. Algae are *Chaetoceros calcitrans, C. muelleri, Isochrysis* sp. (T. iso), *Pavlova pinguis* and *Stichococcus* sp.

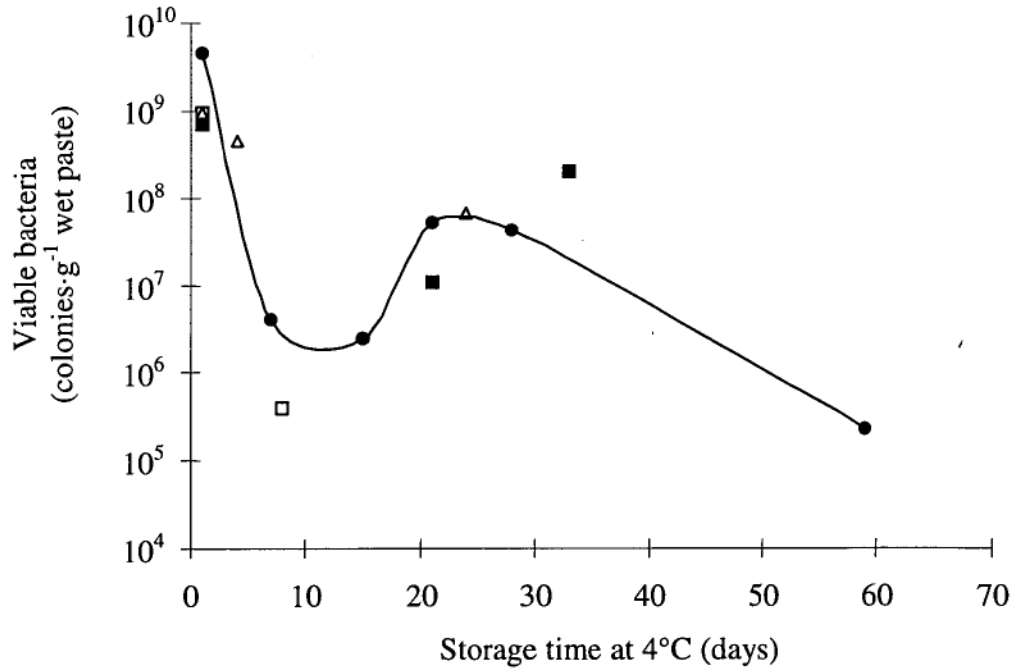


Figure 5.15 Levels of viable bacteria in four pastes (■, □, Δ and ●) of *Thalassiosira pseudonana* stored for extended periods at 4°C.

5.3 v. Ascorbic Acid Concentrations in Algal Pastes

The ascorbic acid (AA) concentration of algal pastes was highly variable, both between pastes of the same and different species (Table 5.8).

Table 5.8 The average and range of ascorbic acid levels initially measured in pastes from a range of microalgae. Sample size (n), varied between species due to the frequency of centrifuge paste trials on selected target species (*Thalassiosira pseudonana* and *Isochrysis* sp.).

Algal species	Sample size	Ascorbic acid ($mg \cdot g^{-1}$ wet paste) Range	Average	CV (%)
Diatoms				
Thalassiosira pseudonana	n=4	0.37-0.65	0.48	25.5
Chaetoceros calcitrans	n=2	0.93-1.04	0.98	8
Chaetoceros muelleri	n=1		0.28	
Prymnesiophytes				
Isochrysis sp. (T. iso)	n=5	0.55-2.22	1.14	60.5
Pavlova pinguis	n=1		0.34	
Chlorophytes				
Stichococcus sp.	n=1		1.02	

AA was measured in algal pastes ($mg \cdot g^{-1}$ wet paste) that were stored for extended periods at 4°C as an indication of the integrity of the pasted cells. A representative diatom (*Chaetoceros calcitrans*), prymnesiophyte (*Isochrysis* sp. (T. iso)), and chlorophyte (*Stichococcus* sp.) paste were examined. Pastes were dispensed into eppendorf tubes and on designated days an individual tube was frozen in liquid-N_2 for later analysis.

The initial AA concentration of the *C. calcitrans* paste was 1.04 $mg \cdot g^{-1}$ paste (Figure 5.16). The AA concentration of this paste fell to <0.05 $mg \cdot g^{-1}$ by day 30, according to the linear equation;

$$AA\ (mg \cdot g^{-1}) = -0.0339x + 1.049 \qquad r^2 = 0.99$$

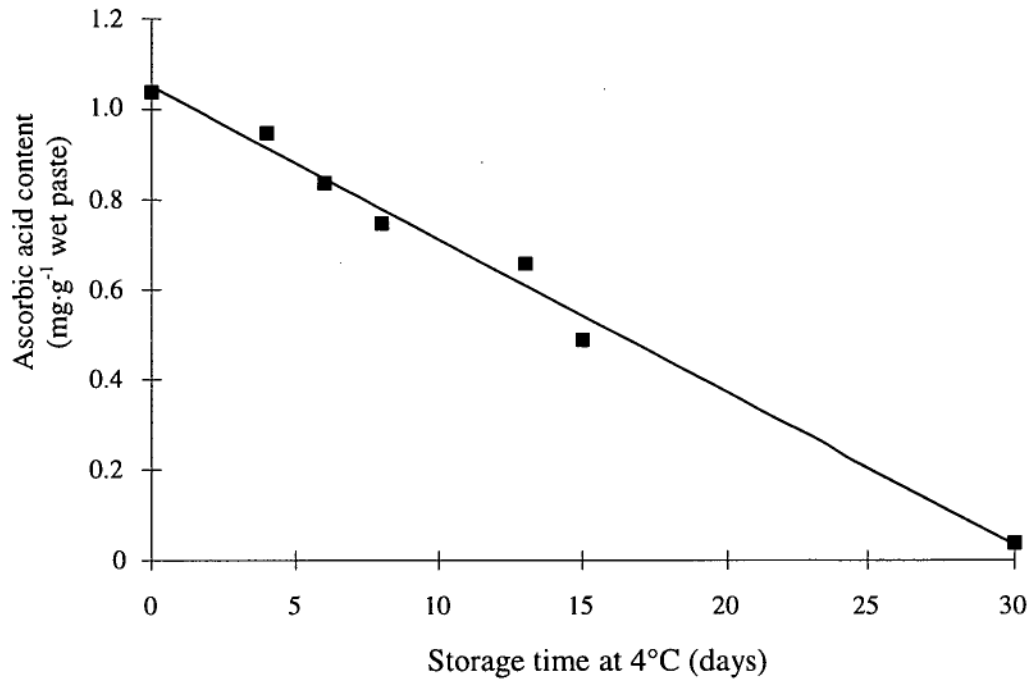


Figure 5.16 Changes in the ascorbic acid content of a paste of *Chaetoceros calcitrans* occurring during storage at 4°C. Data points are average values of duplicate analysis of individual paste samples.

Two pastes of another diatom *T. pseudonana*, had their AA concentration measured over time (Figure 5.17). The AA concentration also decayed in a linear relationship;

$$AA\ (mg \cdot g^{-1}) = -0.0057x + 0.589 \qquad r^2 = 0.81$$

$$AA\ (mg \cdot g^{-1}) = -0.0053x + 0.337 \qquad r^2 = 0.74$$

Although the two pastes had different initial AA concentrations, comparison of the regression lines using a General Linear Model showed no significant difference between the slopes ($F_{1,8} = 0.06$, $P = 0.819$). The rate of loss of AA for the *T. pseudonana* pastes was less than for *C. calcitrans* with projected depletion on day 103 and day 63 respectively. However, resuspension of a sample of paste (day 49) followed by centrifugation and analysis of the pellet showed that only 9% of the AA was still measurable in the collected cells.

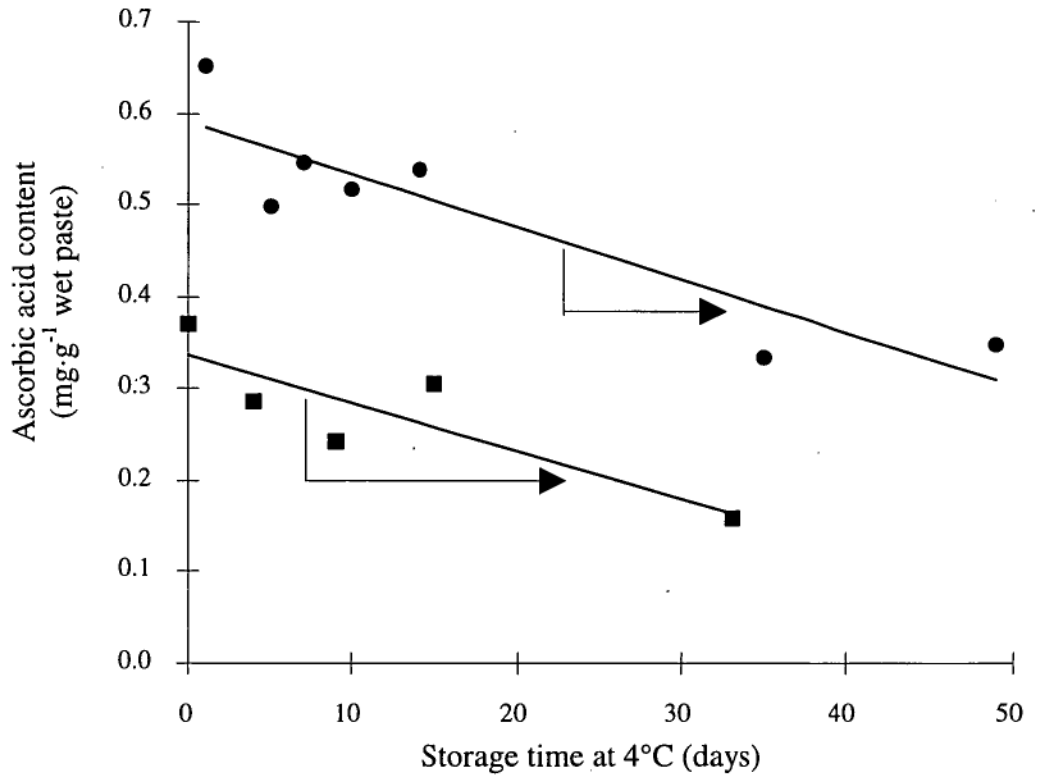


Figure 5.17 Changes in the ascorbic acid content, occurring with time, of two *Thalassiosira pseudonana* pastes stored at 4°C. Pastes were fed to juvenile Pacific oysters with the arrows showing the period that each paste was used in the feeding experiment. Data points are average values of duplicate analysis of individual paste samples.

The initial AA concentration of the *Isochrysis* sp. (T. iso) paste was 2.22 mg·g^{-1} paste (Figure 5.18). The AA content of this paste fell to depletion by day 41, according to the polynomial equation;

$$AA\ (mg \cdot g^{-1}) = 0.0011x^2 - 0.100x + 2.222 \qquad r^2 = 1.00$$

The AA content of the *Stichococcus* sp. paste increased by 90% from 1.02 mg·g^{-1} paste to 1.95 mg·g^{-1} paste during the first 38 days of storage. On day 81 it was 1.41 mg·g^{-1} paste, still 38% more than the initial level (Figure 5.19). Regular analysis beyond day 81 was not undertaken but a single analysis on day 312 showed only negligible (0.04 mg·g^{-1} paste) AA still remaining. For another chlorophyte isolate CS-436, a paste sample taken on day 167 showed an AA content of 0.65 mg·g^{-1} paste. Resuspension of

the sample in seawater, then centrifugation and analysis of the algal pellet showed that 90% of the AA was retained in the cells.

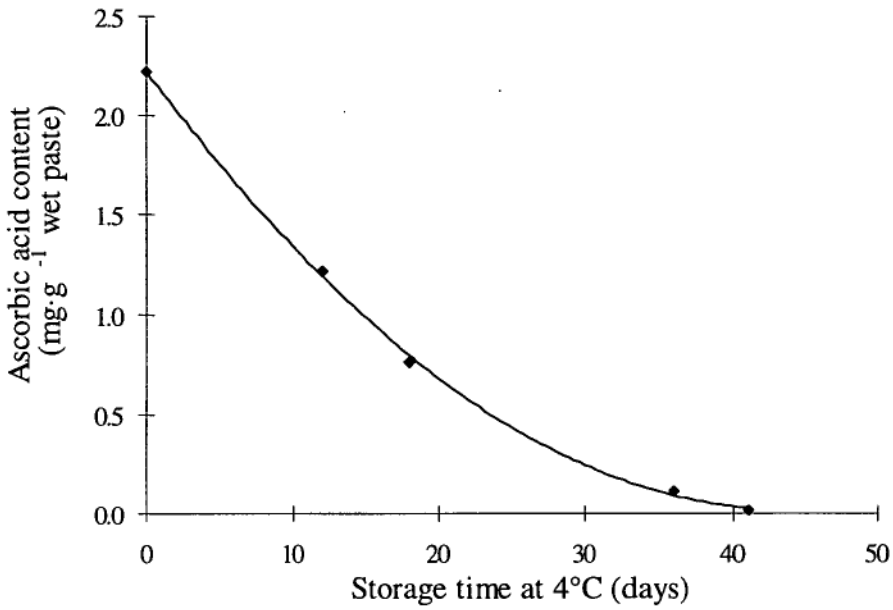


Figure 5.18 Changes in the ascorbic acid content of a paste of *Isochrysis* sp. (T. iso) occurring during storage at 4°C. Data points are average values of duplicate analysis of individual samples.

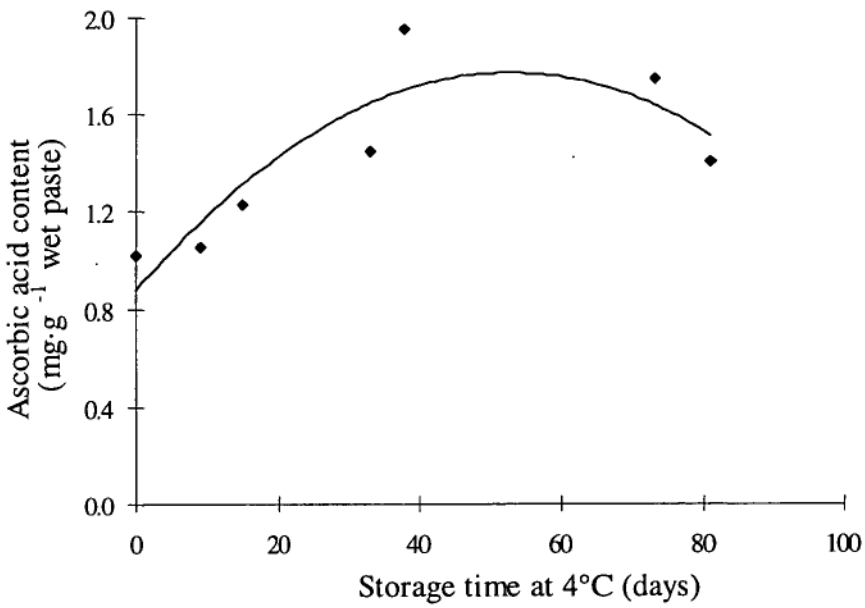


Figure 5.19 Changes in the ascorbic acid content of a paste of *Stichococcus* sp. occurring during storage at 4°C. Data points are average values of duplicate analysis of individual paste samples.

5.3 vi. Protein Concentrations in Algal Pastes

The protein concentration in three of the 4 pastes of *Thalassiosira pseudonana* (Paste 1, 2 and 3; Table 5.3) used in Feeding Experiment 1, were periodically measured during storage at 4°C for up to 70 days (Figure 5.20). The three pastes had a similar initial protein content of 4.69 ± 0.21 $mg \cdot g^{-1}$ wet paste. All pastes then lost protein on storage, most rapidly during the first 21 d and then more slowly until the end of sampling at day 70. A comparison using a General Linear Model of the regression lines during the first 21 days, showed no significant difference between the slopes ($F_{2,7} = 1.35$, $P = 0.319$).

Protein was lost at an average rate according to the equation;

$$\text{Protein } (mg \cdot g^{-1} \text{ wet paste}) = -0.063 * \text{Storage time (days)} + 4.819 \qquad r^2 = 0.93$$

Samples of one of the pastes were also stored at -20°C and protein measured on the corresponding days that 4°C samples were analysed; day 0, 28 and 57. Protein in the frozen samples was stable falling only 4.5% by day 28 and 6.7% by day 57.

One paste sample, analysed on day 68 of storage, was also analysed after resuspension in seawater and re-collection of the cells by centrifugation. The protein content of the paste had fallen 47% from its initial value to be 2.43 $mg \cdot g^{-1}$ and after resuspension it lost 30% of this to be 1.72 $mg \cdot g^{-1}$.

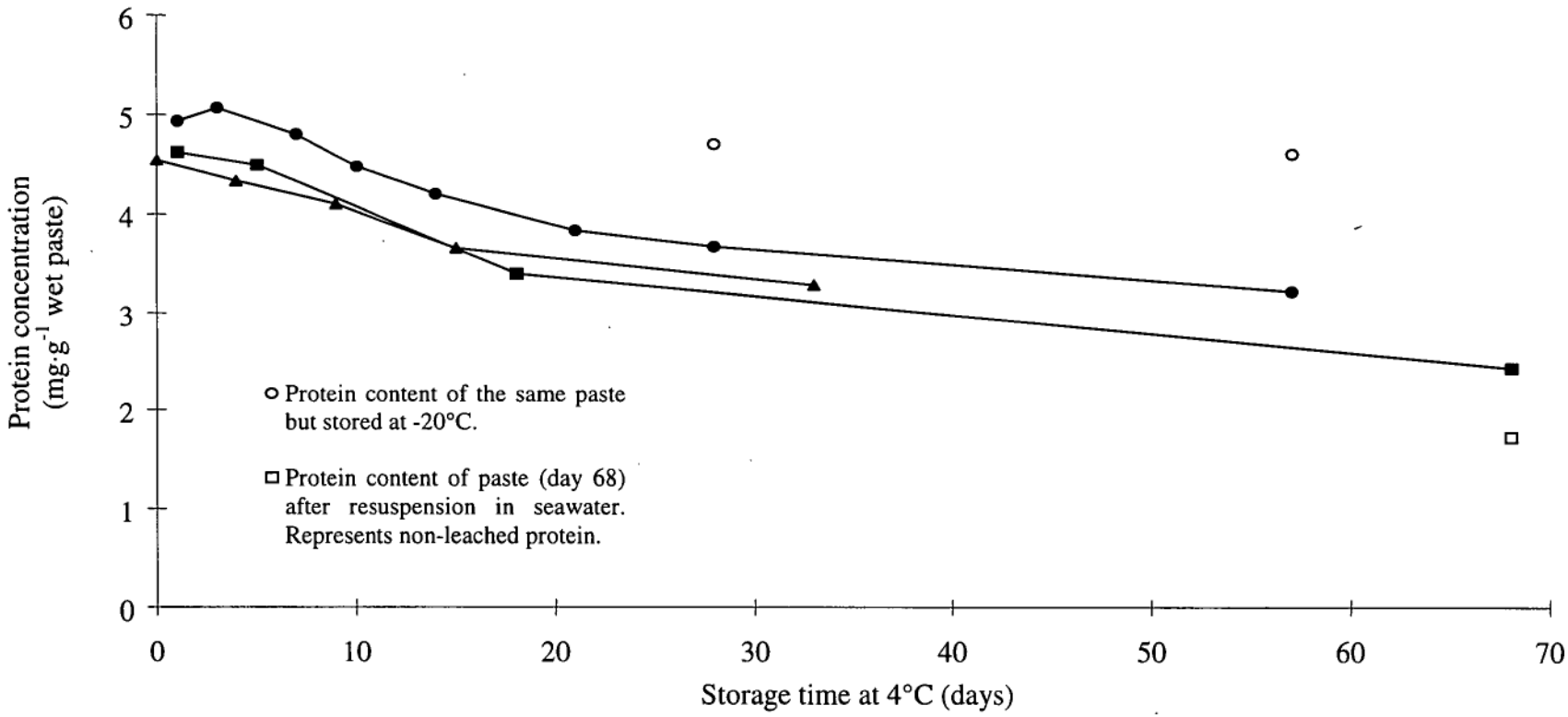


Figure 5.20 The protein concentration of pastes of *Thalassiosira pseudonana* stored for extended periods at 4°C. For one paste, protein values of two similar samples stored at -20°C are presented. One sample of paste, stored at 4°C, was analysed for total protein and a subsample was resuspended in seawater and the residual, non-leached protein was measured.

5.3 vii. Algal Paste Feeding Experiments

5.3 viia. Feeding Experiment 5.1. Nutritional Value vs Storage Time

Over the 18 d experiment, the instantaneous growth rate (k) of oysters (determined from DW and AFDW) were variable (Figure 5.21). An ANOVA on square root transformed data showed highly significant differences between diets when measured as DW ($F=105$, $P<0.001$) or AFDW ($F=225$, $P<0.001$). Fisher's pairwise comparisons showed all diets to be significantly ($P=0.05$) different from each other. The live algal diet was by far the most effective with an average instantaneous growth rate (AFDW) of $0.090 \cdot d^{-1}$, equating to a 414% increase in AFDW. In comparison, the best paste diet (0.5 - 2.5 weeks old) supported a growth rate of $0.034 \cdot d^{-1}$ or an 86% increase in AFDW. Older pastes supported lower growth with a rates of $0.021 \cdot d^{-1}$ (48% increase) for the 2.5 - 5 week old paste and $0.017 \cdot d^{-1}$ (36% increase) for the 11 - 13.5 week old paste. The unfed control oysters had a static dry weight but, lost 6.8% of their organic content (AFDW $k = -0.004 \cdot d^{-1}$).

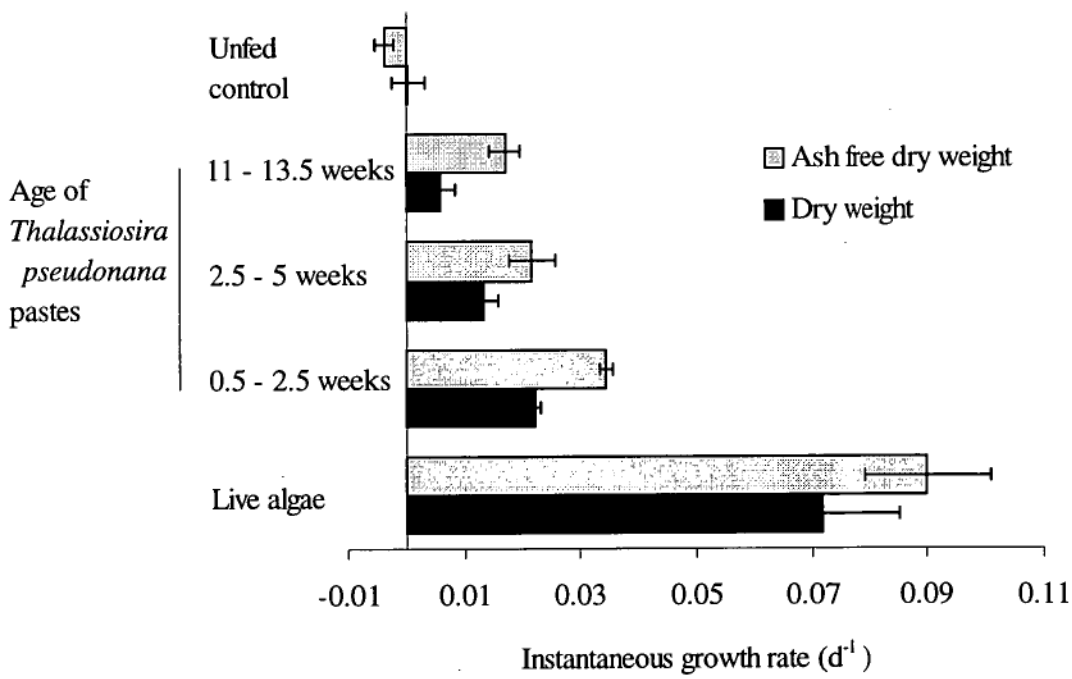


Figure 5.21 Comparison of oyster instantaneous growth rates due to different experimental diets of *Thalassiosira pseudonana*. Diets included three algal pastes of different age and a live algal diet. Error bars are ± 1 S.D. (n=5). All treatments are significantly different.

5.3.viib. Feeding Experiment 5.2. Blended Paste:Live Algae Diets

Over the 21 d experiment, the instantaneous growth rate (k) of oysters (determined from DW and AFDW) were variable (Figure 5.22). An ANOVA on the data showed highly significant differences between diets when measured as DW ($F=164$, $P<0.001$) or AFDW ($F=285$, $P<0.001$).

Fisher's pairwise comparisons showed all diets to be significantly ($P=0.05$) different from each other. The 100% live algal diet gave the highest average instantaneous growth rate (AFDW) of $0.055 \cdot d^{-1}$, equating to a 202% increase in AFDW. The 100% paste diet (0.5 - 2.5 weeks old) supported a growth rate of $0.031 \cdot d^{-1}$ or an 88% increase in AFDW. The blended diets gave growth rates in between those of the 100% live and 100% paste diets. A growth rate of $0.045 \cdot d^{-1}$(147% increase) was produced from the 60:40 paste:live diet and a $0.039 \cdot d^{-1}$(117% increase) rate for the 80:20 paste:live. The unfed control oysters had a small gain in dry weight ($k=0.005 \cdot d^{-1}$, 10% increase) but, lost 11% of their organic content (AFDW $k= -0.006 \cdot d^{-1}$).

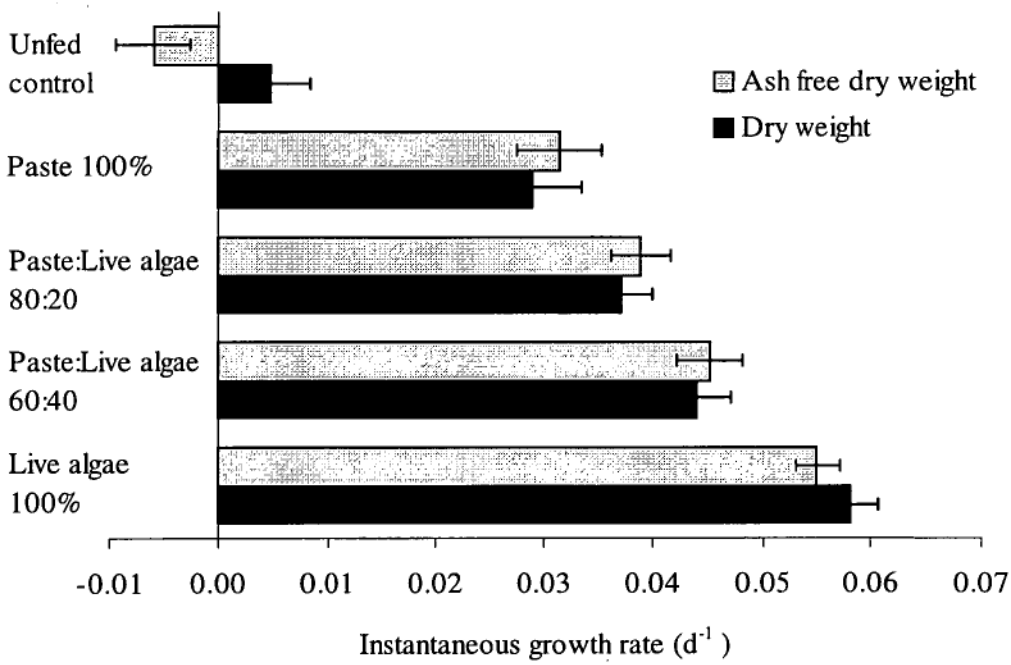


Figure 5.22 Comparison on oyster instantaneous growth rates due to different diet formulations of live and pasted *Thalassiosira pseudonana*. Error bars are ± 1 S.D. (n=5). All treatments are significantly different.

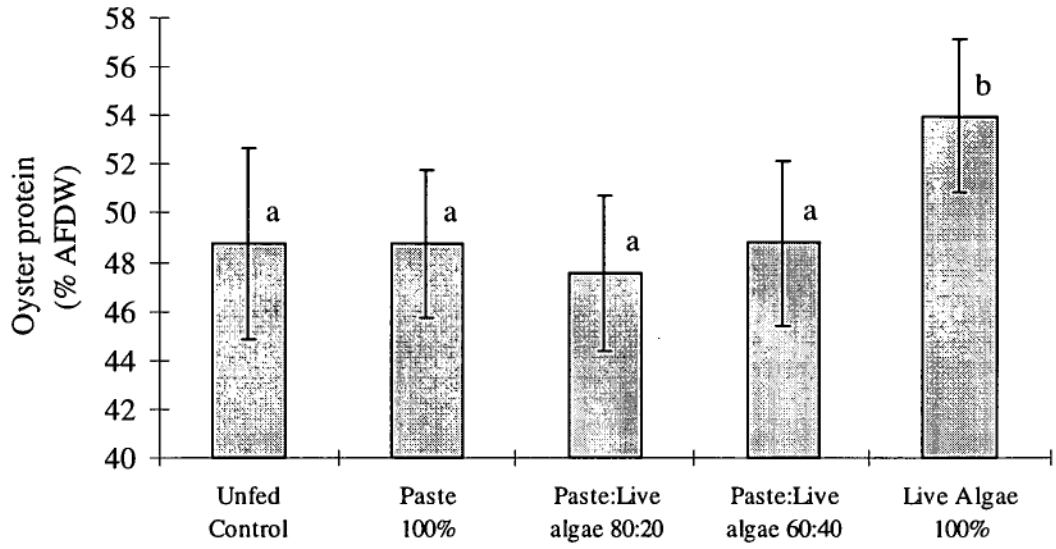


Figure 5.23 The percentage protein composition of oysters fed experimental diets of *Thalassiosira pseudonana*. Error bars are ± 1 S.D. (n=5). Bars without a common letter are significantly different (P<0.05).

Analysis of the proximate composition of oysters at the end of the trial showed diet related differences in oyster composition. An ANOVA for oyster percentage protein showed significant differences between diets (F=2.88, P<0.05). Fisher's pairwise comparison showed that the only significantly different result was for oysters fed 100% live algae (Figure 5.23). Their percentage protein (54% AFDW) was higher than all other treatments which had an average protein content of 48% AFDW.

Oyster percentage carbohydrate were also significantly different between diets (F= 6.51, P<0.005). Fisher's pairwise comparison showed no significant (P=0.05) difference between oysters from the unfed control, 100% paste and 80:20 paste:live diets which had an average carbohydrate of 8.70% AFDW (Figure 5.24). Oysters fed the 60:40 paste:live and 100% live algal diet had an average 10.60% carbohydrate which was significantly greater than the other diets.

To obtain enough lipid for analysis, subsamples of oysters from each replicate in a diet were bulked and a single result obtained for each diet (Figure 5.25). Therefore, differences between treatments are reported as apparent and statistically unsubstantiated. The unfed control oysters had the lowest percentage lipid (8.30%

AFDW). Oysters fed the 100% paste and 80:20 paste:live diets had the highest lipid content of 20.24% and 20.60% respectively. Lipid levels in oysters fed the 60:40 paste:live and 100% live diets were lower at 17.76% and 16.60% AFDW.

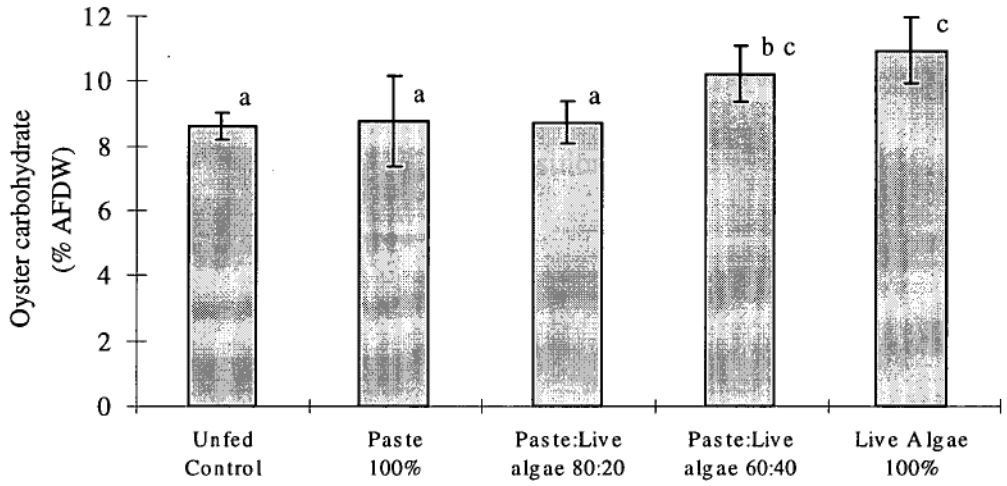


Figure 5.24 The percentage carbohydrate composition of oysters fed experimental diets of *Thalassiosira pseudonana*. Error bars are ± 1 S.D. (n=5). Bars without a common letter are significantly different (P<0.05).

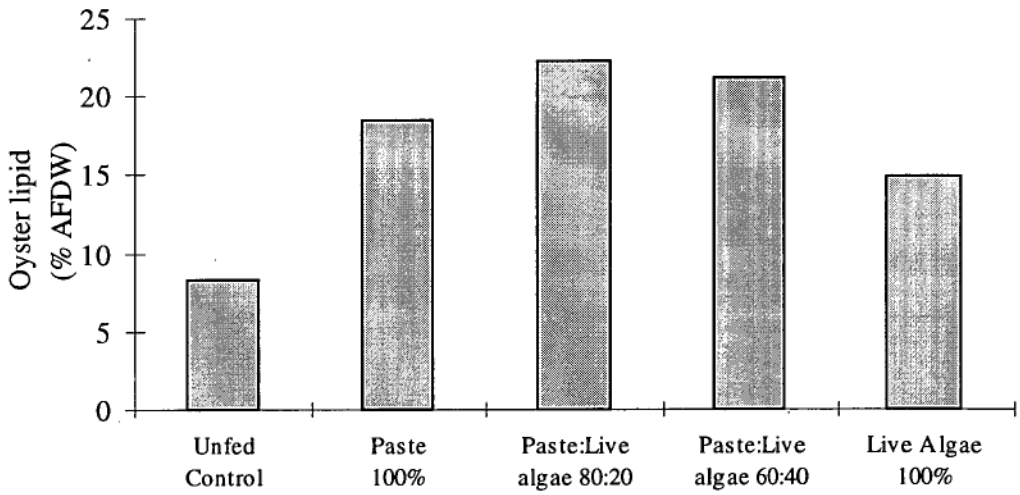


Figure 5.25 The percentage lipid composition of oysters fed experimental diets of *Thalassiosira pseudonana*.

5.4 *Discussion*

Centrifugation was a successful method to concentrate microalgae for the production of algal pastes. Green algal species withstood the process the best and included the prasinophyte *Tetraselmis suecica,* and small, tough chlorophytes species such as *Stichococcus* sp. and isolate CS-436. However, species more nutritious to oysters were damaged by centrifugation and deteriorated with storage.

Centrifugation is a commonly used to concentrate microalgal cultures for the production of algal pastes (Watson 1986; Donaldson, 1991; Nell and O'Connor, 1991; Molina Grima *et al.*, 1994; Lubzens *et al.*, 1995). For short (10-15 min) centrifugation cycles, a chlorophyll *a* mass balance showed that it was an efficient method to concentrate a range of algal species. The harvest efficiency measured from the difference in chlorophyll *a* concentrations of the feed and effluent streams, showed a typical efficiency of 80-85% at 1.1 $L \cdot min^{-1}$. This was confirmed from measuring chlorophyll *a* collected in the centrifuge bowl (paste and liquid concentrate). Centrifugation concentrated microalgae over 2000-fold resulting in a thick paste with a cell concentration of 10^8-10^9 $cell \cdot g^{-1}$ wet paste and average moisture content of 75% for a range of algal classes. These physical properties of algal pastes are typical of other published results and appear independent on the model of centrifuge used for their production. Lubzens *et al.*, (1995) produced pastes of *Nannochloropsis* with a 60-70% moisture content and Tredici *et al.*, (1996) produced pastes of *Isochrysis galbana* with an average 72% moisture content.

As a sole diet for juvenile Pacific oysters (*Crassostrea gigas*), algal pastes of *Thalassiosira pseudonana* were shown to be nutritionally inferior (Figure 5.21). There were two main conclusions from this feeding experiment. Firstly, that a live algal diet was nutritionally superior to all tested algal pastes, providing an instantaneous growth rate (AFDW) of $0.090 \cdot d^{-1}$. This is comparable to published growth rates for juvenile oysters fed nutritionally balanced diets (Enright *et al.*, 1986a). The best of the paste diets was the youngest paste (0.5 to 2.5 weeks) with an oyster instantaneous growth rate of $0.034 \cdot d^{-1}$. The second conclusion was that the nutritional values of the algal pastes

deteriorate with increasing storage time. Oyster growth rates fell to $0.021 \cdot d^{-1}$ for pastes of 2.5 to 5 weeks of age and to $0.017 \cdot d^{-1}$ for a paste of 11 to 13.5 weeks. The algal pastes resuspended to single cells and were ingested by the oysters. The poor growth rates therefore reflect a loss in nutritional value of pasted algae.

Biochemical analysis of algal pastes indicated a range of factors that could combine to result in a loss of nutritional value. An early indication of the viability of pasted cells was the detection of chlorophyll degradation products (phaeopigments). For pastes of *T. pseudonana* stored at 4°C, the average chlorophyll *a* concentration fell from 4.2 to 2.7 $mg \cdot mL^{-1}$ over the first three weeks of storage. During this period, scans of chlorophyll extracts revealed a progressive shift in the Soret peak of chlorophyll from a λ_{max} of 430nm as for native chlorophyll *a* to a λ_{max} of 410nm, characteristic of phaeopigments. The apparent loss of total chlorophylls is likely to be an artefact of its transformation into phaeopigments, which have a lower extinction coefficient (Jeffrey *et al.*, 1997) than native chlorophyll. By day 24 there was no residual λ_{max} 430nm peak. PMDC observed a similar trend in pastes of *Isochrysis galbana* where phaeopigments increased during storage (4°C) with an associated loss of measured photosynthetic activity.

Measurements of ascorbic acid (AA) in algal pastes also indicated a loss of cell viability and integrity. The initial AA concentration of algal pastes was highly variable, both between pastes of the same and different species. Brown and Miller (1992) measured the AA content of eleven species of microalgae used in mariculture and found levels to be variable between species (0.11 to 1.62% AA) and to be affected by their phase of growth. For *Chaetoceros calcitrans* they reported a range of 0.17 to 0.36% DW, a little below the 0.50% measured in a culture concentrated to a paste in this study. The AA concentration of the pasted *C. calcitrans* was $1.04 \ mg \cdot g^{-1}$ (wet paste), equating to a 3340 fold concentration during centrifugation and comparable to the 2800-fold concentration in the measured chlorophyll for the same culture.

For *C. calcitrans, T. pseudonana* and *Isochrysis* sp. (T. iso), AA concentration began to fall immediately after concentration to a paste and continued to fall until it was

undetectable. Loss was linear in pastes of the two diatoms and followed a polynomial relationship for *Isochrysis* sp. (T. iso). The rate of loss was influenced by the method of storage with a slower rate in pastes sealed in syringes than for samples in eppendorf tubes.

The protein concentration in pastes of *T. pseudonana* also reduced with increasing storage time. Three pastes had a similar initial protein content (4.7 $mg \cdot g^{-1}$ wet paste) which fell linearly and maximally during the first 21 d of storage. This coincides with the period that chlorophyll is degraded to phaeopigments and may indicate the general loss of intracellular integrity with the associated release of enzymes, which could digest proteins.

Microscopic examination of the centrifuged algal pastes showed clear signs of structural damage to cells. For *T. pseudonana* there was an increase in the size and shape of the cells and for *Isochrysis* sp. (T. iso) there was a retraction of the cytoplasm. These observations indicate that cells were leaking metabolites. Measurement of the total AFDW of pastes of *T. pseudonana* used in Feeding Experiment 5.1, showed the four pastes to have a similar AFDW of 10.5% (CV = 4.4%). After resuspending the pasted cells in seawater there was a loss in measured AFDW that was greater in older pastes. For recently pasted cells (4 d) around 25% of the AFDW could leach from the cells on resuspension and that cell integrity deteriorated in older pastes so that by 11 weeks, 57% of AFDW could leach. Leaching has major implications for the assumed availability to oysters of all measured cell contents. The AA concentration of two of the pastes used in Feeding Experiment 5.1 was measured over time (Figure 5.17) and at least half of the initial AA was still present in the pastes during the period that they were fed to oysters. However, after resuspension of one of the pastes (49 days old) only 9% of the AA was still measurable in the cells. Even in a much younger paste (16 days old), only 16% of AA remained after resuspension. It must be assumed that levels would have fallen further during the 24 h that the oysters fed. Protein was also found to leach from the same pastes with 30% lost on resuspension of a 68 d old paste.

The feed rate for each oyster diet was set at a constant DW, after resuspension of pasted cells, and so was independent of leaching. However, what had leached from the cells would have a major influence on the nutritional value of the remaining dietary AFDW. Essential and easily assimilated compounds such as vitamins, free sugars, amino acids and small peptides would all be prone to leaching, leaving mainly structural proteins and polysaccharides for assimilation by the oysters. As leaching increases with the age of the algal paste, their nutritional value declines. Since the live algal diet was so superior to even the youngest paste that leached around 25% AFDW when resuspended, then the leached organics must be essential or comprise the major nutritional value of the algae.

If the poor nutritional value of pasted algae is due to leaching of an essential nutrient such as a vitamin, feeding it as part of a mixed diet with live algae could compensate and improve the nutritional value of the paste component. In Feeding Experiment 5.2, algal pastes (0.5 to 2 weeks old) were fed to juvenile oysters with treatments including blended diets of live and pasted cells. The live algal diet was again the best giving an oyster instantaneous growth rate (AFDW) of $0.055 \cdot d^{-1}$ compared to $0.031 \cdot d^{-1}$ for the paste (100%) fed oysters, equal to 130% more growth over the period of the experiment (Fig. 5.22). Blending the paste and live algae did improve the nutritional value of the paste component. Assuming that the growth from the blended diets would equal the sum of the proportion of 100% live and paste growth (60:40 Paste:Live = 60% of the 100% paste growth + 40% of the 100% live growth) then additional growth was taken to be the improvement in nutritional value of the paste component due to partial compensation for its nutritional deficiencies. This indicated that blending 20% live algae increased the nutritional value of the paste by 10% and by 27% at a 40% live algal blend. The percentage improvement in the nutritional value of the paste was related to the percentage of live algae according to the polynomial equation;

$$\text{Percentage improvement} = 0.0105x^2 + 0.253x \qquad r^2 = 1$$

Although the nutritional value of the paste was improved the relationship shows that the nutritional value of the paste can never be fully compensated for.

Oysters fed high levels of algal pastes had a lower carbohydrate content which was partially compensated for at a 40% live algal blend. This indicates that oysters fed high levels of algal paste are energy starved and have a low glycogen reserve. This could be a result of low calorific value of the diet or to cessation of growth due to a nutrient deficiency.

Nell and O'Connor (1991) fed oyster larvae (*Saccostrea commercialis*), algal pastes (7 to 14 d old) produced using a cream separator centrifuge. They found flagellates to be damaged by centrifugation and storage, but diatoms to be unaffected. When *C. calcitrans* paste was fed as a sole diet it was not significantly different from the live algae culture, but *P. lutheri* paste was a poor diet compared to its live culture control. However, when fed in combination *Pavlova lutheri* and *C. calcitrans* pastes produced greater growth than the equivalent live algal diet. Watson *et al.*, (1986) also found the diatoms *C. calcitrans* and *T. pseudonana* to withstand centrifugation the best and that the flagellates *P. lutheri* and *Isochrysis galbana* to be severely damaged. They found the quality of pastes to be variable between batches and that the nutritional value declined with storage at 4°C to be significantly less than live algae within 2-3 weeks. Other authors have also reported the rapid loss of nutritional value of refrigerated pastes (Brown, 1995; Montaini *et al.*, 1995; Molina Grima *et al.*, 1994; Donaldson, 1991). In a series of feeding experiments using *C. gigas* larvae (2 and 8 d old), Muller-Feuga *et al.*, (1996) found a diet of *I. galbana* paste caused high larval mortality and poor growth. This effect was greater in the younger larvae (80% mortality) than the older larvae (50%). They noted a high degree of cell damage in resuspended pasted-cells.

In contrast to the loss by leaching of AA found in some pastes in this study, Brown (1995) found that 4 week old (4°C) pasted cells of *C. calcitrans* retained 92% of their AA intracellularly after resuspension. However, the AA concentration fell 29% over the same period. This compares to a 90% drop in AA concentration over a similar period in a *C. calcitrans* paste in this study. The difference reflects the level of damage caused to cells during centrifugation. SEM revealed no visible damage to pasted and resuspended cells in Brown's study where cells were concentrated in laboratory centrifuges. The continuous-flow Alfa Laval cream separator used in this study has been

shown to physically damage diatoms and this is likely to result in a more rapid oxidation of the AA and leaching upon resuspension.

As discussed, the ability of microalgae to withstand centrifugation and storage as a paste is variable between species. However, when comparing published results on the characteristics of pastes and their nutritional value the method of centrifugation must be considered. Algae concentrated by laboratory centrifuges at relatively low speed (Brown, 1995; Molina Grima *et al.*, 1994) are subjected to much lower gravitational and shear forces than algae concentrated in high speed continuous centrifuges (Tredici *et al.*, 1996; Montaini *et al.*, 1995). Flynn and Al-Amoudi (1988) found that *Phaeodactylum tricornutum* cells lost a significant portion of their intracellular free amino acids when centrifuged at 4000 g.

In this study concentrating using the high-shear Alfa Laval cream separator damaged highly nutritious microalgal species including diatoms and prymnesiophytes. The same species may have suffered less damage if concentrated in alternative centrifuges. However, in contrast to diatoms and prymnesiophytes some algal species were little affected by centrifugation using the cream separator. In particular, small chlorophytes such as *Stichococcus* sp. and isolate CS-436 (*Chlorella*-like) showed no visible damage after centrifugation and resuspension of the pasted cells and produced pastes that were very stable. A paste of isolate CS-436 stored for 136 days showed no shift in the peaks in chlorophyll extracts that would indicate degradation. Resuspension of a sample of the same paste after 167 days storage (4°C) showed the cells to have a high level of integrity with only 10% of AA leaching from the cells. The prasinophyte, *Tetraselmis suecica* was affected by centrifugation with a change in cell shape and the loss of flagella. However, it also produced a stable paste that lost only 10% of chlorophyll *a* after 257 d storage with no detection of chlorophyll degradation products. Although these algae were better candidates for concentration by centrifugation, they are nutritionally inferior as diets for oysters (Langdon and Waldock, 1981; Enright *et al.*, 1986a).

Algal pastes of these more centrifuge-resistant species could be useful dietary components for other aquaculture species. Pastes of *Tetraselmis,* could find application in diets for *C. virginica* where Wikfors *et al.*, (1996) found high lipid strains of *Tetraselmis* spp. supported significantly faster oyster growth than an equivalent ration of *Isochrysis* sp. (T. iso). As part of a binary diet with *Skeletonema costatum*, *T. suecica* was a nutritious diet for juvenile *Saccostrea commercialis* (O' Connor *et al.*, 1992). Spray-dried *T. suecica* has also found application in diets for juvenile Manila clams (Laing and Millican, 1991) and in penaeid larviculture (Biedenbach *et al.*, 1990). Algal pastes of freshwater *Chlorella* have enabled the culture of the marine rotifer *Brachionus rotundiformis* at high density, 10000 to 30000 rotifers·mL^{-1} (Yoshimura *et al.*, 1996).

To produce algal concentrates of better nutritional quality an alternative low-shear concentration process was required. The process would need the capacity to harvest large volume of algae and to be cost-effective. In chapter 6 methods to concentrate microalgae based on flocculation are developed and the nutritional quality of the flocculated concentrates are compared to centrifuged algal concentrates.

Chapter Six

Production of Algal Concentrates by Flocculation

Chapter 6

Production of Algal Concentrates by Flocculation

6.1 *Introduction*

Production of algal concentrates using centrifugation involves exposing cells to high gravitational forces. The use of simple centrifuges such as cream separators also results in very high shear forces due to the design of the distributor. Large amounts of air are also present which causes cavitation in the culture feed stream resulting in additional stresses on the cells. In Chapter 5, it was shown that these forces combine to severely damage all but the very smallest and toughest algal species. Algal species suitable as oyster diets were damaged from centrifugation with major losses in their nutritional value. An alternative low shear concentration method was required.

Flocculation is the coalescence of finely divided suspended solids into larger loosely packed conglomerates. It can be a low shear, chemical based method to concentrate algae. Flocculation is widely used in industry to remove suspended solids. It has applications in the clarification of drinking water, the treatment of sewage and the concentration of minerals in mining operations. It has also been used in the harvesting of microalgae for subsequent extraction of metabolites such as beta-carotene from *Dunaliella salina.*

The exact chemistry of flocculation is not always well defined but involves two stages. The first stage is the aggregation of suspended solids into larger particles. This results from the interaction of the flocculant with the surface charge of the suspended solids. The second stage involves flocculation of the aggregates into large flocs that settle out of suspension. This occurs from the precipitation of the flocculant. For an iron flocculation process the ion, Fe^{+3} binds to the negative surface charge of the algal cells causing aggregation. The Fe^{+3} then precipitates as hydroxides that bind the aggregates into large flocs.

A wide range of high molecular weight polymers are commercially available that can be cationic, anionic or non-ionic and can replace metal salts as flocculating agents. These polymers can be natural products such as chitin but most are formulations of polyacrylamide.

Flocculation is typically used in fresh water systems where it is most efficient. Because the process initially involves the interaction of charges it works best when the flocculant and the suspended solid are the dominant charges. In saline water, the presence of large amounts of ions interferes with the process and much larger doses of flocculant are required to induce flocculation.

Generally most flocculation applications produce a floc that is a waste stream or it is processed further to extract elements from within it. As a method to produce algal concentrates for aquaculture feeds, the floc must be de-flocculated to release the trapped algal cells.

In this chapter, I discuss methods to produce algal concentrates by flocculation and to assess their nutritional value for juvenile Pacific oysters.

6.2 *Materials and methods*

6.2 i. Microalgal cultures

Microalgae from four algal classes were assessed for their ability to be concentrated by flocculation (Table 6.1).

Table 6.1 Microalgae species assessed for their ability to be concentrated by flocculation.

Algal class	Species and CSIRO culture code		Flocculation method
Diatoms	*Attheya septentrionalis*	CS-425	Ferric and pH
	Chaetoceros calcitrans	CS-178	pH
	Chaetoceros muelleri	CS-176	pH
	Nitzschia closterium	CS-5	pH
	Thalassiosira pseudonana	CS-173	Ferric and pH
	Skeletonema sp.	CS-252	pH
Prymnesiophyte	*Isochrysis* sp. (T. iso)	CS-177	Ferric
Prasinophyte	*Tetraselmis suecica*	CS-187	pH
Cryptophyte	*Rhodomonas salina*	CS-24	pH

Culture volumes of 1.5 L to 10 L were used in assessment trials. Large volume (100-500 L) cultures of selected algal species were flocculated to determine the scale-up potential of the process. Small volume cultures (1.5-10 L) were grown as outlined in section 2.1. Larger volume cultures were grown in polyethylene bags, a polycarbonate tank or open fibreglass tanks. Culture conditions were similar to 10 L cultures, but were less regulated than those in controlled environment growth cabinets. Temperature was partially regulated by airconditioning in the range 20 ± 2°C and illumination was by cool white fluorescent lights, either continuously or 12:12 h light/dark cycle. Metal halide lamps illuminated tank cultures (500-1000 L). Modified f/2 media (Table 2.1) was used for all cultures.

6.2 ii. Flocculation

6.2 iia. Ferric Induced Flocculation

Initial experiments with ferric-induced flocculation were conducted in 250 mL beakers using a magnetic stirrer for mixing. These were scaled-up to 10 L, 120 L and 500 L flocculations using aeration for mixing. Stock solutions of $FeCl_3 \cdot 6H_2O$ (0.1M) and polyelectrolyte (0.05% LT-25, Allied Colloids) were used to flocculate algal cultures. For beaker experiments the stock solutions were diluted 1:5-1:10 with 0.01M HCl for $FeCl_3 \cdot 6H_2O$ and 1:10 with Milli-Q water for the polyelectrolyte. For larger volume flocculations the stock solutions were used undiluted.

The $FeCl_3 \cdot 6H_2O$ was added first at the region of maximal mixing above the point of aeration. It was added slowly so that the whole culture volume was exposed to Fe^{+3} before it precipitated as hydroxides. For 250 mL volumes, addition of $FeCl_3 \cdot 6H_2O$ took 5-10 seconds but for 500 L volumes it took ~5 minutes. After addition of $FeCl_3 \cdot 6H_2O$, mixing was continued until small flocs were visible; then the polyelectrolyte was added rapidly and the mixing reduced. When large flocs formed (~1 minute) the mixing was stopped and the flocs allowed to settle under gravity. Surface water was then siphoned off and the settled floc collected and stored at 4°C. After 24 h at 4°C the floc settled further and surface water was again removed.

6.2 iib. pH-Induced Flocculation

As the pH of seawater is increased to pH 10.6-11 a buffering region is encountered where hydroxides of dissolved metal salts precipitate, trapping algal cells within the hydroxide flocs. Precipitate continues to form over a wide buffering region forming low density, bulky flocs that are slow to settle and have an increasing alkalinity. Algal flocculation was best achieved with the use of minimum chemicals (NaOH) and a low bulk of floc.

Initial experiments with pH-induced flocculation were run in 250 mL beakers using a magnetic stirrer for mixing. These were scaled-up to 10 L, and 500 L flocculations using aeration mixing. Stock solutions of NaOH (1M) and polyelectrolyte (0.05% LT-25, Allied Colloids) were used to flocculate algal cultures. For beaker experiments,

both stock solutions were diluted 1:5-1:10 with Milli-Q water. For larger volume flocculations the NaOH stock solution was diluted 1:2 with potable water and the polyelectrolyte used undiluted.

The NaOH was added first at the point of maximal mixing and the pH measured. It was added slowly so that there was a uniform increase in culture pH. Addition of the caustic was critical because if localised high pH is formed then beads (~5 mm) of hydroxides precipitate, raising culture alkalinity but not flocculating algal cells. For 250 mL volumes, addition of NaOH took 5-10 seconds but for 500 L volumes it took 5-10 minutes. As the buffering region is entered there is a shift in the clarity of the culture as the white precipitate is formed, but flocs are difficult to see. After addition of NaOH, mixing was reduced and the polyelectrolyte was added. When large flocs formed (~1 minute) the mixing was stopped and the flocs allowed to settle under gravity. Surface water was then siphoned off and the settled floc collected and immediately de-flocculated (section 6.2 iii.).

6.2 iii. De-flocculation and Resuspension

For use of flocculated algae as feed for filter feeding bivalves, the flocs must be disrupted to release the algal cells. Ferric floc was de-flocculated by addition of Na-EDTA. The iron content of floc was measured (Section 6.2 v.) and Na-EDTA solution (pH 4.5) added on an equal molar basis. The floc was mixed for 5 min before being centrifuged (170 g, 5 min) to recover cells. The yellow, iron-rich supernatant was discarded and the collected cells resuspended in sterile seawater.

The pH-induced floc was de-flocculated immediately after collection by adjusting the pH of the floc slurry to pH 7.5-8 using concentrated HCl. Acid was added slowly at the point of maximal mixing until the pH stabilised in the desired region. The floc slurry darkens in colour as the bulk of the white precipitate dissolves leaving small (<1 mm) aggregates of densely packed cells. These were settled overnight at 4°C and any supernatant removed.

To resuspend flocculated algal concentrates for feeding to juvenile oysters, the required quantity of concentrate was dispensed into a 50 mL plastic tube half-filled with seawater. The tube was gently shaken to until clumps disappeared and then the suspension was diluted in seawater and fed to oysters. Samples of suspension were filtered for DW and AFDW (section 2.1 i.).

6.2 iv. Measurement of Flocculation Efficiency and Mass

In small volume (250 mL) trials, efficiency of flocculation was measured after centrifuging the flocculated culture in 50 mL tubes (10 g, 2 min). Centrifugation of non-flocculated culture showed that free cells were not concentrated during the centrifugation cycle. An aliquot of supernatant was taken to measure chlorophyll *a* (section 2.2 i.) and efficiency determined by comparison to the initial culture. When flocculating with ferric chloride, an aliquot was also taken to measure residual iron. The floc pellets were combined in a graduated glass tube and centrifuged (670 g, 3 min) to measure packed floc volume. For ferric floc the pellet was then resuspended and an aliquot taken to measure the concentration level. To measure flocculation efficiency in larger volumes, chlorophyll *a* was measured in an aliquot of the supernatant after gravity settlement of the flocs.

6.2 v. Iron Analysis

Iron was measured using a colorimetric method adapted from Vogel (1978). The method measures iron as the red complex formed between Fe^{+2} and 1,10 ortho-phenanthroline. Ferric iron can also be measured by reduction with hydroxylamine hydrochloride. The reaction does not appear to be affected by high salinity.

To redissolve iron hydroxides, the sample pH was adjusted to 0.8-1.0 using concentrated HCl. Samples were then filtered through glass-fibre (GF/C) filters or centrifuged. Depending on the expected iron concentration, the samples were diluted in 0.01 M HCl according to the following rates:

Dilution	Working range ($\mu M Fe^{+3}$)		
No dilution	10	to	300
1 in 5	50	to	1500
1 in 10	200	to	6000
1 in 100	100	to	30000

For assay blank and duplicate standards, 5.0 mL Milli-Q water was pipetted into 3 x 15 mL glass test tubes. For test sample, 3 x 5.0 mL aliquots were pipetted into glass test tubes. These represented duplicate sample assays and a standard addition to confirm no interference from the sample media. Other samples were analysed in duplicate. To the duplicate standards and the sample standard addition tube, 100 µL of iron standard (5mM $FeCl_3 \cdot 6H_2O$ in 0.01M HCl) was added and the samples mixed. To all tubes, 4 mL 10% (w/v) hydroxylamine hydrochloride solution was added, then 1 mL of 2M acetate buffer (pH 4.0) followed by 4 mL of phenanthroline solution (0.25% w/v). Samples were mixed after addition of each reagent. Samples were left for at least 10 minutes at room temperature, mixed, then their absorbance measured at 508 nm against the assay blank.

The average standard absorbance of all the standards and standard addition tubes was calculated and the total iron concentration determined from the formula;

$$\text{Fe concentration } (\mu M) = \frac{\text{Sample Absorbance x 100 x Dilution Factor}}{\text{Average Absorbance of Standards}}$$

The linear range of the assay was 10 - 400 µM Fe (Absorbance 0.05-2.2).

The rate of colour formation was severely inhibited by the presence of high levels of EDTA. Samples containing EDTA were left overnight to fully develop colour, with no loss of colour in assay standards. Inclusion of sample standards confirmed the completion of colour development.

6.2 vi. Assessment of Flocculated Concentrates and Algal Pastes: Feeding Experiment 6.1.

Flocculated algal diets were assessed for their nutritional value to juvenile Pacific oysters in feeding experiment 6.1, comparing flocculated, centrifuged and live *Thalassiosira pseudonana* diets. Freshly graded juvenile oysters (1500 µm; 1.0 mL packed volume) were dispensed into each of 28 experimental upwellers. Four replicates of each treatment were run (Table 6.2).

Table 6.2 Treatments and method of formulation for diets fed to juvenile Pacific oysters in feeding experiment 6.1.

Treatment	Diet	Method of formulation
1	Unfed control	
2	Live *T. pseudonana*	
3	Centrifuged *T. pseudonana*	Laboratory centrifuged concentrate
4	Centrifuged *T. pseudonana*	Super-centrifuged paste
5	Centrifuged *T. pseudonana*	Alfa Laval cream separator paste
6	Flocculated *T. pseudonana*	Ferric flocculated concentrate
7	Flocculated *T. pseudonana*	pH flocculated concentrate

Algae used for all diets were grown in 10 L polycarbonate carboys (section 2.1.), except the Sharples, Super-centrifuge paste. The Super-centrifuge diet was supplied by NSW State Fisheries, Port Stephens, from a centrifuged 1000 L *T. pseudonana* culture (Figure 6.1.). Laboratory centrifuged concentrate was prepared from 25 L of culture centrifuged in a Sorval RT6000 (1500 g, 10 min) and a Sorval RC-5 (1900 g, 10 min) centrifuges. Concentrate was collected from each centrifuge cycle and combined so that all cells had only one, 10 min centrifuge cycle. For the Alfa Laval cream separator paste, 40 L of culture was centrifuged at an average flow rate of 0.9 $L \cdot min^{-1}$. For ferric flocculation the pH of 40 L of culture was raised to pH 9.5 and Fe^{+3} was added to give a final concentration of 230 µM. Polyelectrolyte (0.05% LT-25) was then added (1 $mL \cdot L^{-1}$) and the flocs left to settle overnight at 4°C, then collected. The pH flocculation was induced by addition of 0.5M NaOH to 30 L of culture to give a final [NaOH] of 7 mM.

Polyelectrolyte (0.05% LT-25) was then added (1 $mL \cdot L^{-1}$) and settled flocs were transferred to a measuring cylinder and allowed to settle further overnight at 4°C, then collected. Flocculated and centrifuged diets were 0.5-1 week old at the start of the feeding experiment, which ran for 22 days.

All diets were added daily at a rate of 10 mg DW·upweller^{-1}. The DW and AFDW of each diet were measured daily. On days 8, 15 and 22, a subsample of 50 oysters was removed from each upweller and DW and AFDW determined (section 2.1 ii.). Results were used to calculate instantaneous growth rates for each period (section 2.1 ii.) and diets were assessed by comparing the oyster growth rates from the experimental diets to that of the live algal fed oysters.

At the end of the experiment (day 22), 100 oysters from each upweller were taken and stored at -20°C. Daily samples of the algal diets were filtered (GF/C) and stored at -20°C. These were grouped to form three samples for each diet, representing the feeding periods; day 1-8, day 9-15 and day 16-22. Oyster and grouped algal samples were analysed for protein, carbohydrate and lipid (section 2.2 iii.).

Figure 6.1 The disassembled Sharples, Super-centrifuge used to make the 'Super-centrifuge' algal paste diet. The cylindrical centrifuge bowl (on table top) fits inside the main body of the machine and the cover (on shelf of table) fits on top. Culture is fed in at the base of the unit and cells collect as a paste on the internal surface of the bowl.

6.3 i. Development of Ferric Flocculation

Flocculation efficiency of *Thalassiosira pseudonana* cells increased with increasing addition of Fe^{+3} (Figure 6.3.). Efficiency increased linearly up to 250 µM Fe^{+3} but was highly variable at the lower Fe^{+3} concentrations with a coefficient of variation (CV) of 117% and 62% at 62.5 and 125 µM Fe^{+3}. At 250 µM Fe^{+3} flocculation was more consistent (CV= 6%) and efficient (85%). At higher Fe^{+3} concentrations, 500 and 1000 µM there was only a small increase in flocculation efficiency, reaching 96 and 93% respectively.

The main effect of flocculating with higher Fe^{+3} concentrations was an increase in the mass of the formed floc (Figure 6.2). Over the Fe^{+3} dose range of 0-1000 µM, the mass of formed floc increased linearly according to the equation;

Packed Floc Volume (mL·250 mL^{-1} culture) = 0.0012 [Fe^{+3}] $r^2 = 0.98$

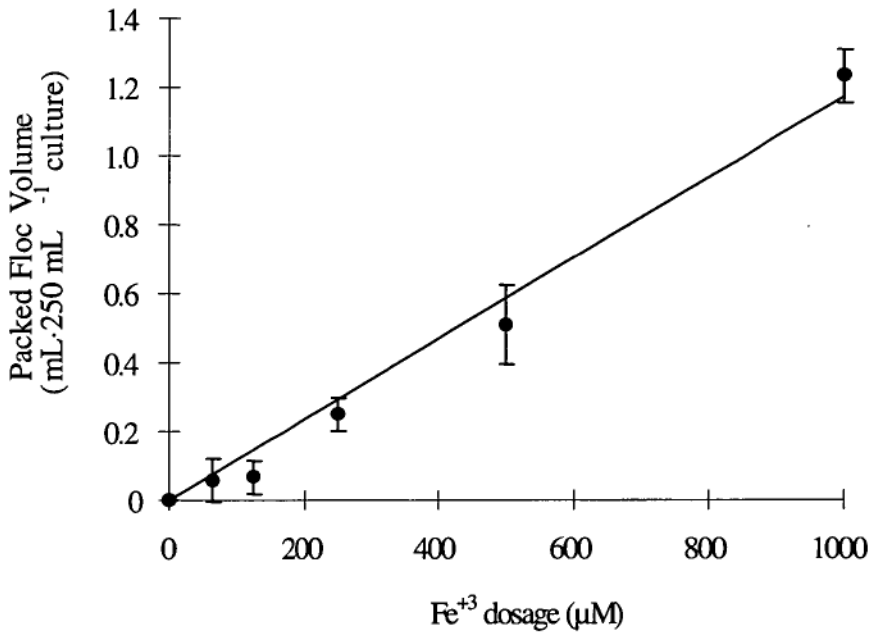
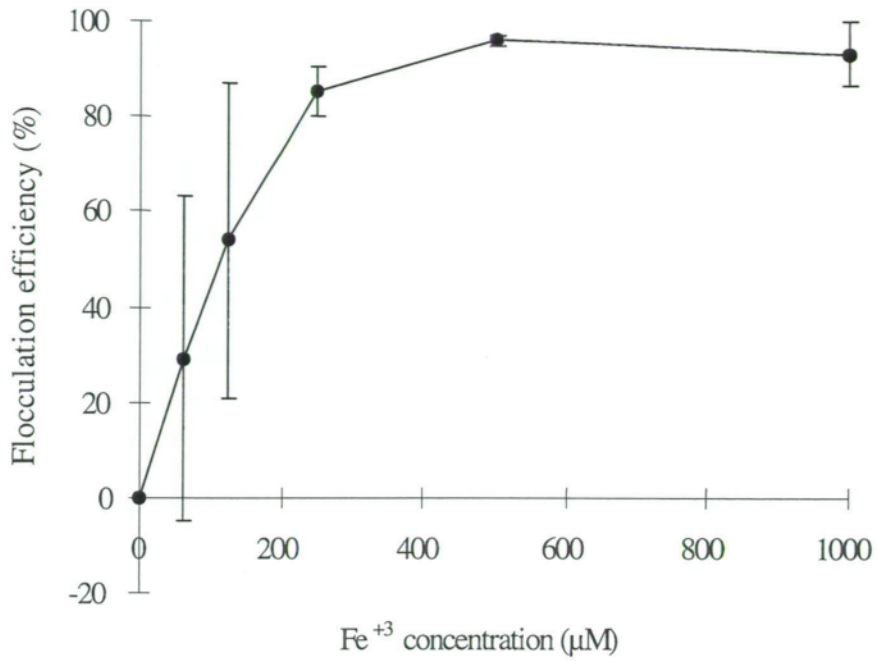


Figure 6.2 The packed volume of floc produced from 250 mL of *Thalassiosira pseudonana* culture flocculated with 0-1000 µM Fe^{+3}. Error bars are ± 1 S.D. (n=3).

A)



B)

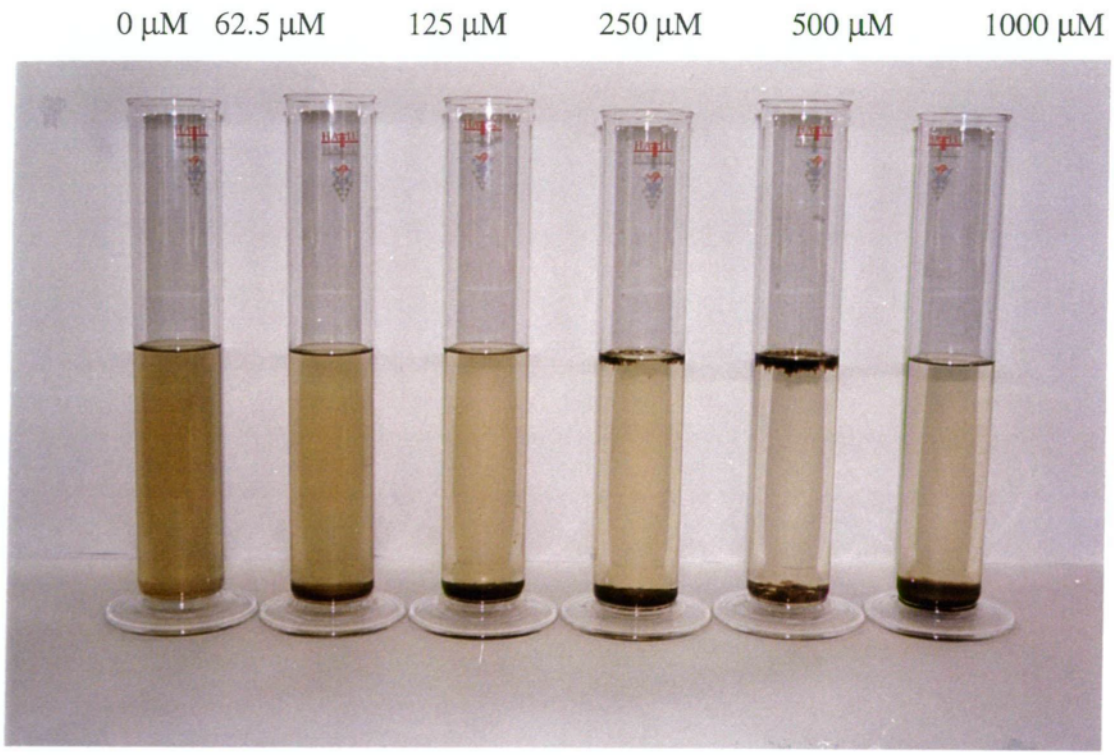


Figure 6.3 A) Flocculation efficiency of *Thalassiosira pseudonana* (250 mL) using increasing concentrations of ferric chloride with polyelectrolyte (0.05%) added at 1 $mL \cdot L^{-1}$. Error bars are ± 1 S.D., (n=3).
B) Photograph of one replicate taken after 1 h, some floc has floated due to photosynthesis-generated gas bubbles.

Flocculation efficiency determined from chlorophyll *a* analysis, increased only slightly at Fe^{+3} doses above 250 µM, but the mass of floc produced increased in a linear relationship. Therefore, flocculating at Fe^{+3} concentrations above 250 µM produced a floc with fewer algal cells per unit of floc. This effect was seen when the flocculation efficiency results shown in Figure 6.3, were combined with measurements of flocculated algal cells per µmole Fe^{+3} (Figure 6.4). Although flocculation was less efficient and reliable at Fe^{+3} concentrations below 250 µM, the recovered flocs were densely packed with algal cells (2.6-5.5 x10^7 cells·µmole^{-1} Fe^{+3}). At 250 µM Fe^{+3}, cells were efficiently flocculated and the floc contains less cells per unit of floc (1.2 x10^7 cells·µmole Fe^{+3}). As more floc mass was produced at 500 and 1000 µM Fe^{+3}, the concentration of algae falls to 4.7 x10^6 and 2.6 x10^6 cells·µmole Fe^{+3}. Further development of Fe^{+3} flocculation was performed using 250 µM Fe^{+3} . At this concentration, algal cells were efficiently harvested using the minimum quantity of chemicals to produce a floc that was densely packed with cells.

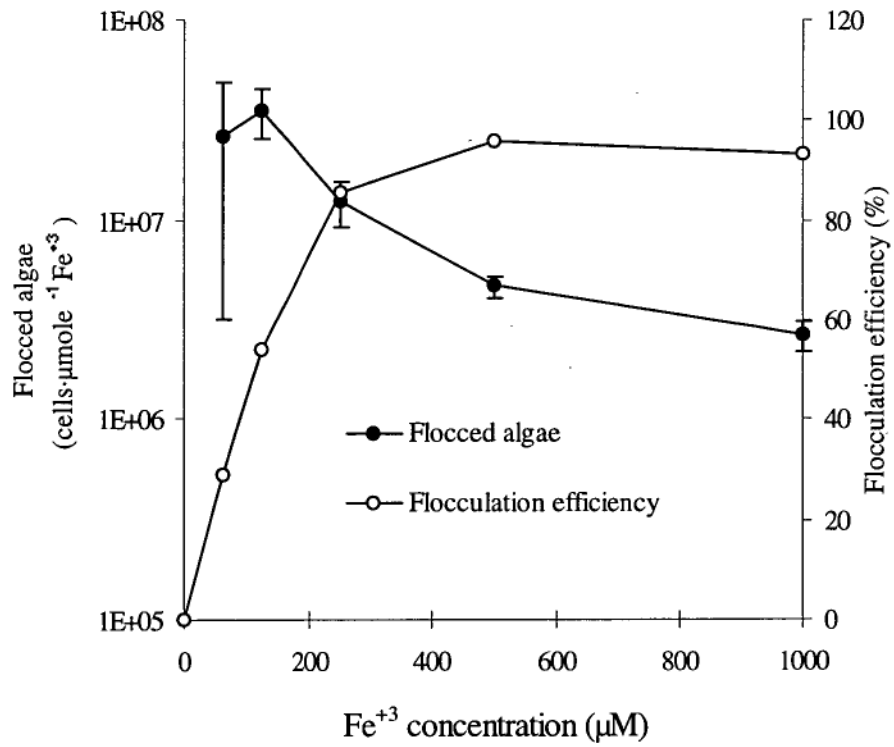


Figure 6.4 Comparison of the efficiency of flocculation (*Thalassiosira pseudonana*, 250 mL) with increasing Fe^{+3} concentration and the density of algal cells in the produced flocs. Error bars are ± 1 S.D. (n=3).

Adjustment of culture pH prior to flocculation showed pH to be a significant factor in determining flocculation efficiency. Cultures of *T. pseudonana* were adjusted to pH 5-10 and flocculated with 250 µM Fe^{+3} (Figure 6.6). Flocculation was highly variable at pH 5 (CV= 53%) with a reduced efficiency of 49%. At pH 6 it was more consistent (CV= 10%) with a minor peak in efficiency (86%). At pH 7 and 8 flocculation remained efficient but, was more variable (average CV= 18%). Variability was reduced at pH 9 and by pH 10 there was a significant increase in efficiency to 97% (CV= 2%).

The added Fe^{+3} may remain in solution or form insoluble, ferric hydroxide floc. Iron analysis showed initial pH determined the partitioning of the added Fe^{+3} between soluble and insoluble phases (Figure 6.5). At pH 5, where harvest efficiency was low, almost 80% of the added Fe^{+3} remained in solution. At pH 6-8 a stable region was encountered where around 60% of the Fe^{+3} was recovered in the flocs. When the pH was increased to over 9, there was an apparent net gain in total iron with just over 100% of added Fe^{+3} being recovered in the floc. This was a result of precipitation of some of the iron that was present as a micronutrient in the growth media.

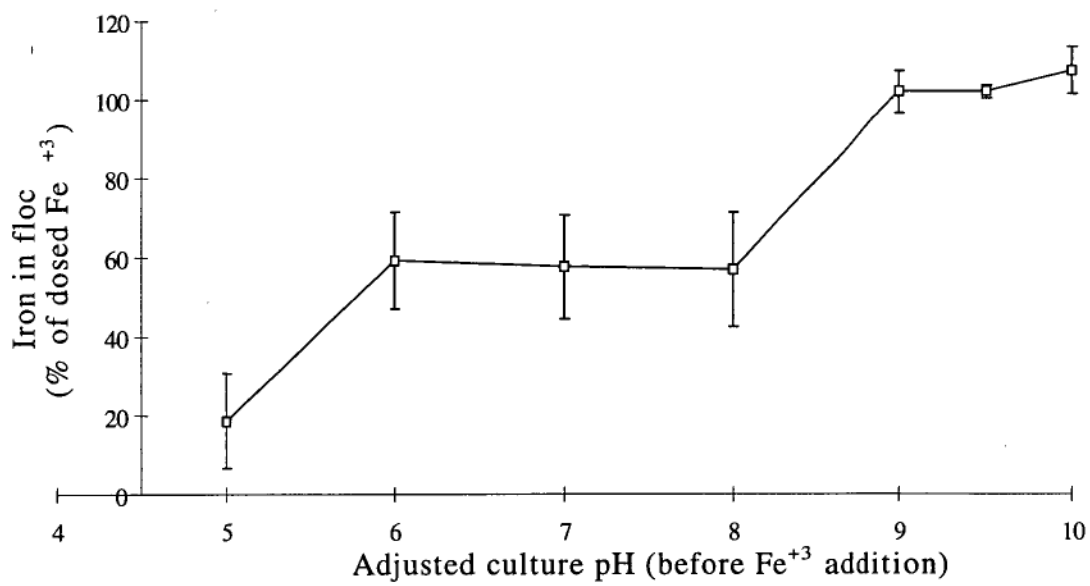
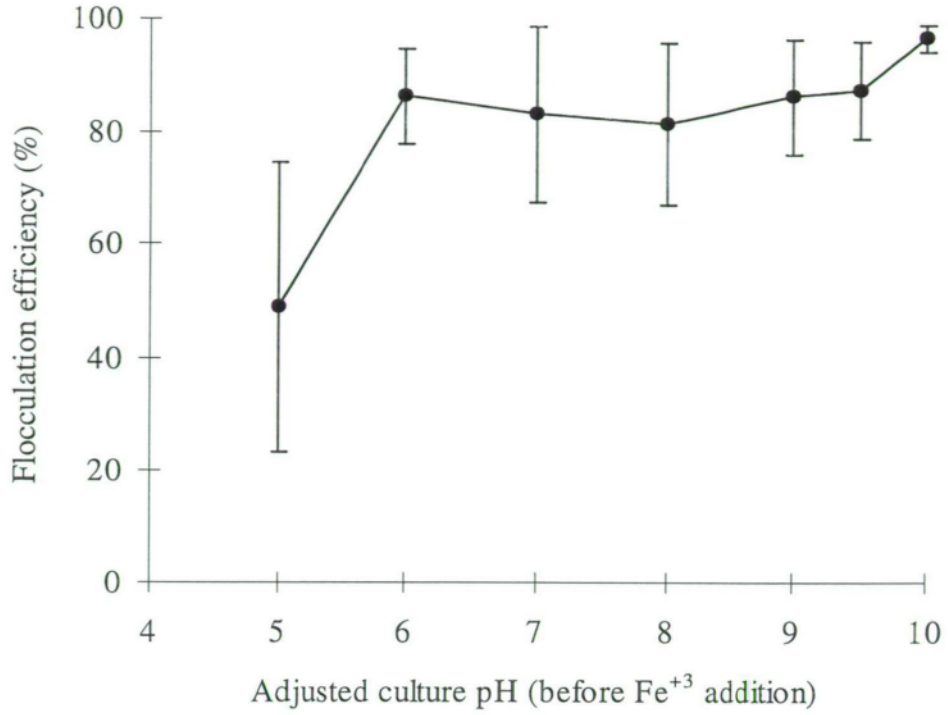


Figure 6.5 Iron recovered in the floc as a percentage of that added during flocculation (250 µM Fe^{+3}) of pH adjusted *Thalassiosira pseudonana* cultures (250 mL). Error bars are ± 1 S.D. (n=3).

A)



B)

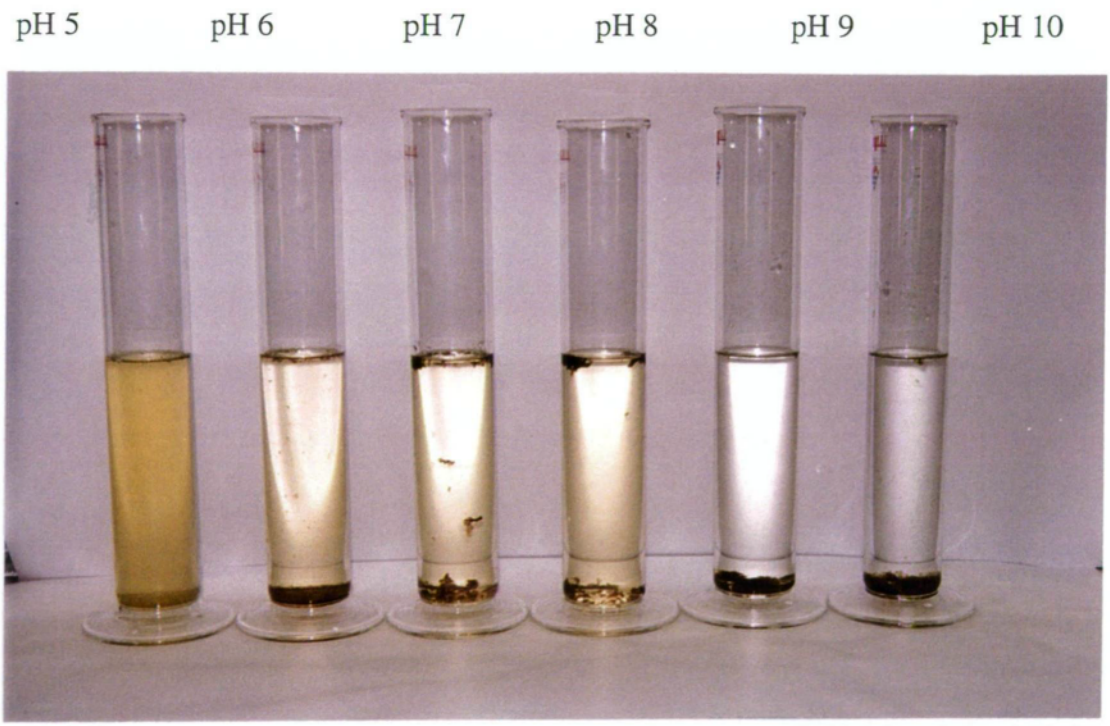
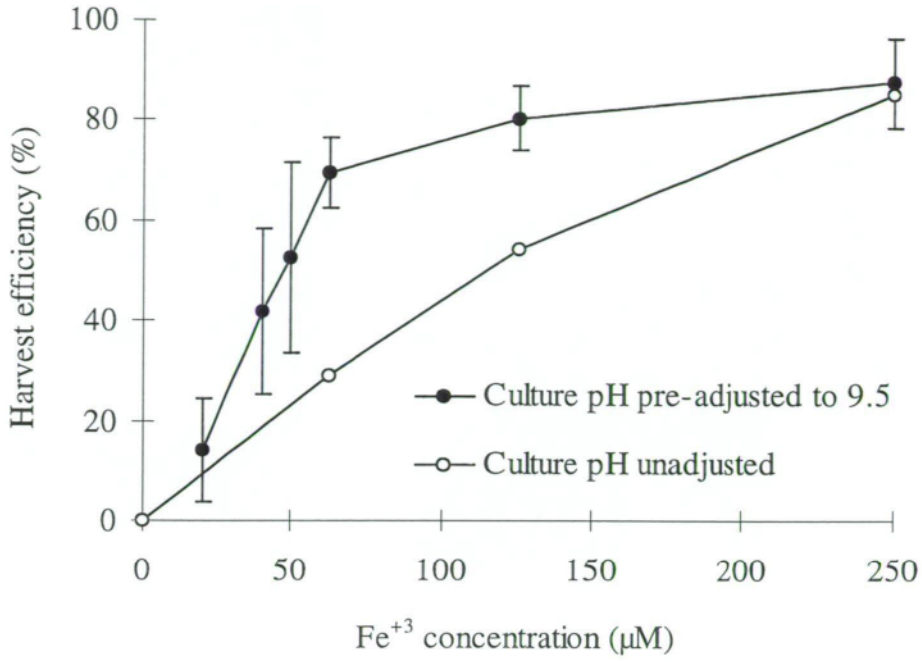


Figure 6.6 A) Flocculation efficiency of *Thalassiosira pseudonana* (250 mL) with pre-adjusted pH. Ferric chloride dosed at 250 µM with polyelectrolyte LT-25 (0.05%) added at 1 $mL \cdot L^{-1}$. Error bars are ± 1 S.D. (n=3).
B) Photograph of one replicate taken after 1 h, some floc floating due to photosynthesis-generated gas bubbles.

Because flocculation efficiency increased at high pH values, flocculation efficiency versus Fe^{+3} concentration was re-evaluated with cultures pre-adjusted to pH 9.5 (Figure 6.7). For cultures with non-adjusted pH, Fe^{+3} concentrations below 250 μM produced variable, inefficient flocculation. However, pre-adjusting the culture to pH 9.5 extended the lower Fe^{+3} limit that produced efficient flocculation from 250 μM to 62.5 μM. At this level, flocculation efficiency was 70% (CV= 10%) compared to 29% (CV= 117%) for cultures without adjusted pH. At Fe^{+3} concentrations below 62.5 μM, flocculation rapidly became inefficient and more variable.

A)



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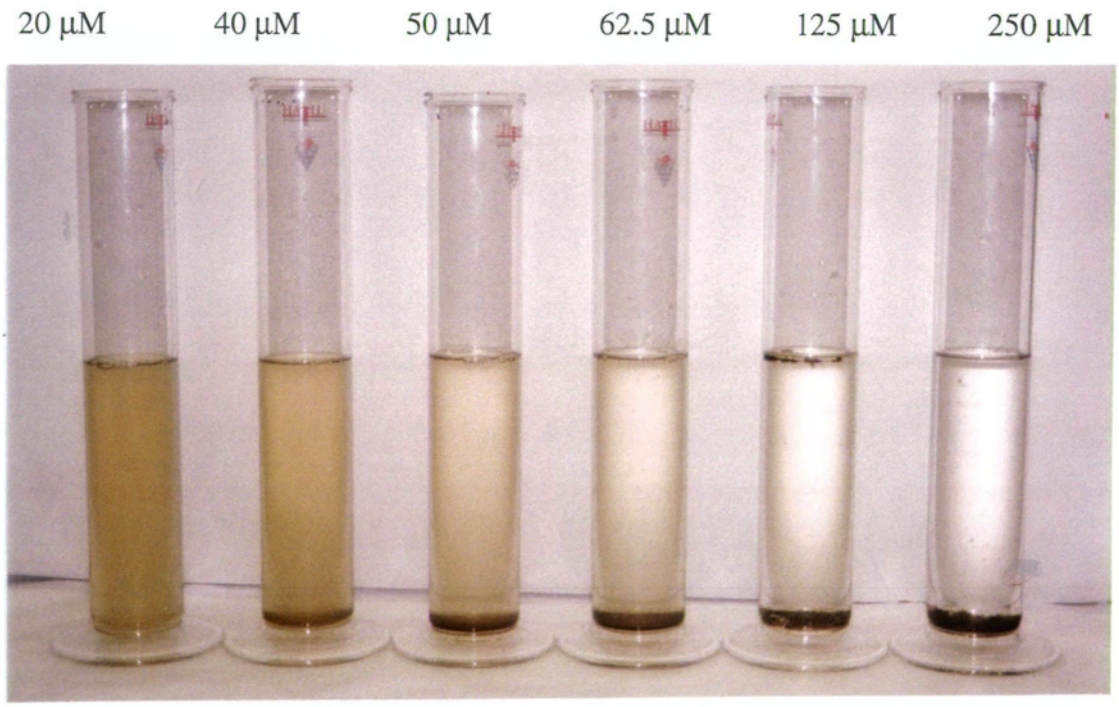


Figure 6.7 A) Flocculation efficiency of *Thalassiosira pseudonana* (250 mL), pre-adjusted to pH 9.5. Ferric chloride concentration from 20-250 µM with polyelectrolyte LT-25 (0.05%) added at 1 $mL \cdot L^{-1}$. Efficiency compared to flocculation of cultures with unadjusted pH. Error bars are ± 1 S.D. (n=3).
B) Photograph of one replicate taken after 1 h, some floc floating due to photosynthesis-generated gas bubbles.

6.3 ia. Ferric flocculation of *Isochrysis* sp. (T. iso)

The efficiency of ferric induced flocculation of *Isochrysis* sp. (T. iso) was evaluated with pre-adjustment of culture pH from pH 5 to 10 and a Fe^{+3} concentration of 250 µM. Flocculation efficiency was poor and variable, but there was a loss of efficiency for cultures pre-adjusted to pH 8 and then an improvement in efficiency at higher pH levels (30-50% efficient at pH 10). For cultures pre-adjusted to pH 9.5 and flocculated with [Fe^{+3}] from 62.5 to 500 µM, the efficiency of algal flocculation increased with increasing Fe^{+3} concentration to reach 31% at a [Fe^{+3}] of 500 µM (Figure 6.8). The percentage of added Fe^{+3} recovered in the floc varied little over the range of Fe^{+3} concentrations (average 88%). Though most of the Fe^{+3} formed flocs, the algal cells were inefficiently flocculated. At higher Fe^{+3} concentrations there was more floc produced and the gradual increase in harvest efficiency was likely a result of more cells being entrapped in the floc and not to a significant improvement in the actual coagulation and flocculation of algal cells.

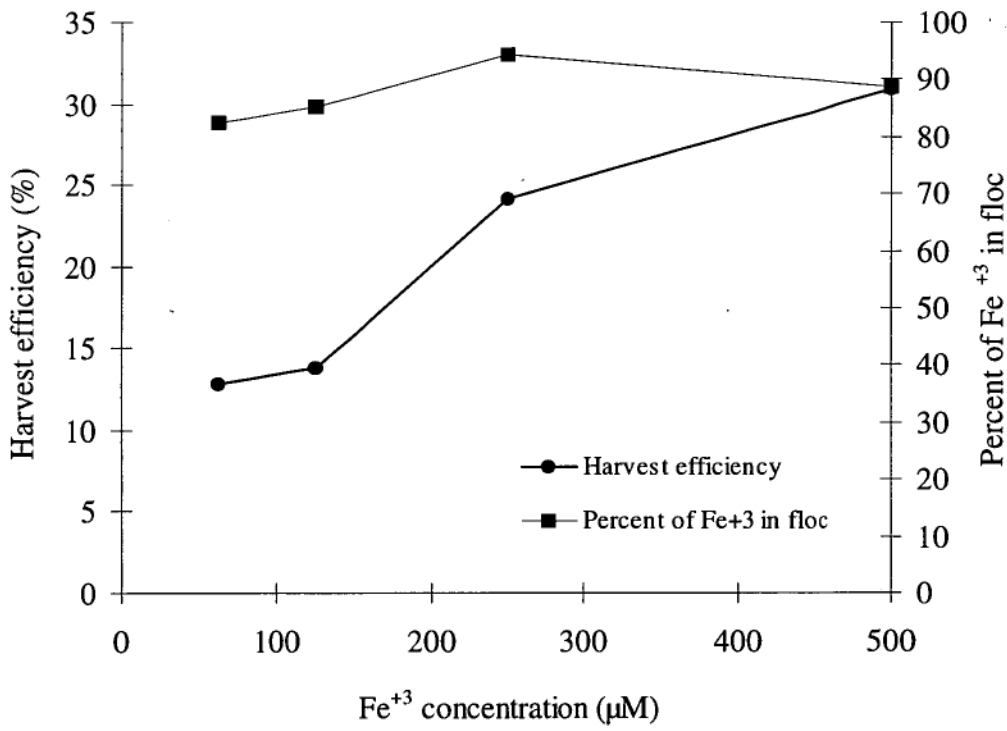


Figure 6.8 The harvest efficiency of *Isochrysis* sp. (T. iso) cultures (250 mL), pre-adjusted to pH 9.5 and flocculated with varying concentrations of Fe^{+3}. The percentage of added Fe^{+3} recovered in the floc is displayed on the secondary y-axis. Values are averages from duplicate treatments.

6.3 ii. Development of pH-Induced Flocculation

When adjusting the pH of cultures during the evaluation of Fe^{+3} flocculation, a buffering region was encountered at pH 10.5-11. In this region, a precipitate formed that entrapped the algae and pH induced flocculation was evaluated as an alternative to Fe^{+3} flocculation.

Formation of the precipitate was a chemical reaction, independent of the presence of algal cells. Titrating coastal and oceanic seawater with NaOH showed the pH to increase rapidly to pH 10.6 following addition of 4 mM NaOH (Figure 6.9). A buffering region was then entered which extended from 4 mM to 90 mM NaOH before the pH increased.

Precipitate started to form after addition on 4 mM NaOH and continued to form in a linear relationship in the buffering region according to the equation (Figure 6.10);

Floc mass (mg DW·250 mL^{-1} seawater) = 9.74[NaOH mM] - 8.71 r^2= 0.99

The precipitate was fine and white and close to neutral buoyancy. Continued addition of NaOH turned the whole water column a milky-white as more precipitate formed. However, the precipitate remained fine and was very slow to settle. Addition of the polyelectrolyte, flocculation enhancer (LT-25) greatly increased the size of flocs and the rate at which they settled. The bulk of the settled floc and hence the concentration factor, reflected the quantity of NaOH added to form the precipitate. Algal harvesting experiments were undertaken to determine the minimum NaOH addition, with the use of polyelectrolyte, that would reliable flocculate cells.

Flocculation efficiency of *Thalassiosira pseudonana* cultures was measured over the NaOH concentration range 3-13 mM (Figure 6.11). Average flocculation efficiency increased according to the polynomial equation;

Flocculation efficiency (%) = $0.172x^3 - 5.297x^2 + 55.10x - 102.8$ r^2= 1.00
where x = [NaOH] mM.

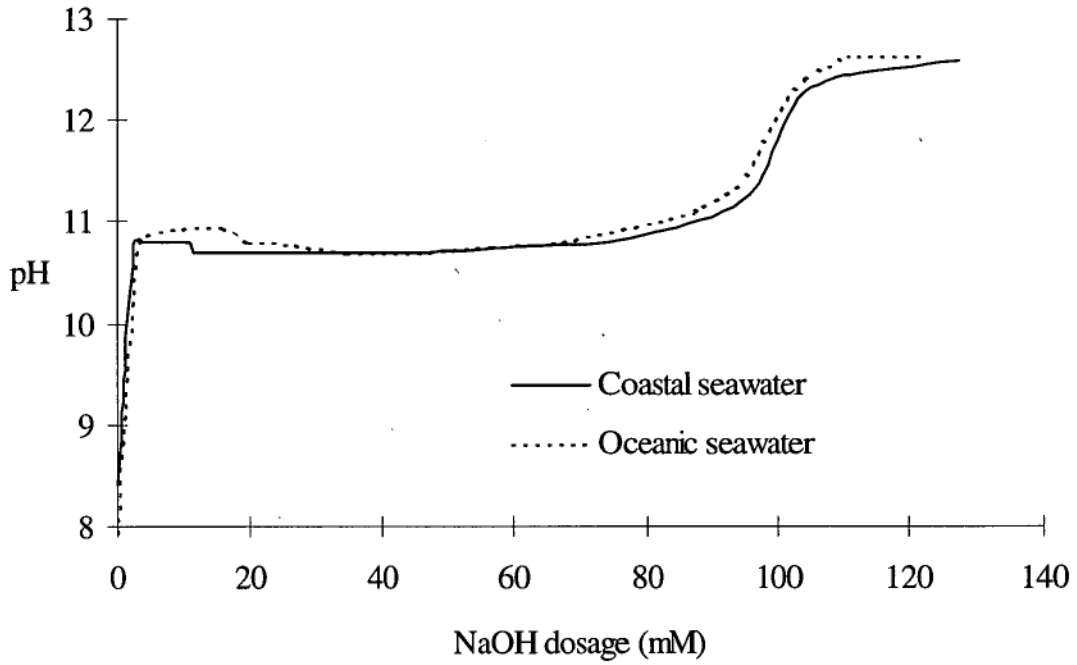


Figure 6.9 Effect on the pH of coastal and oceanic seawater of the increasing addition of NaOH.

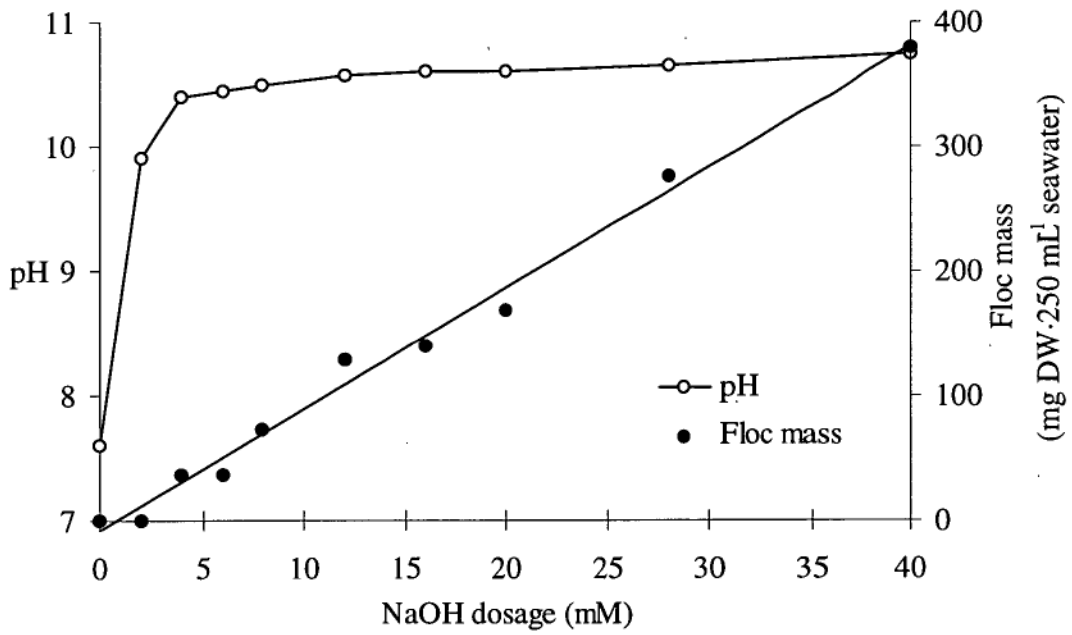
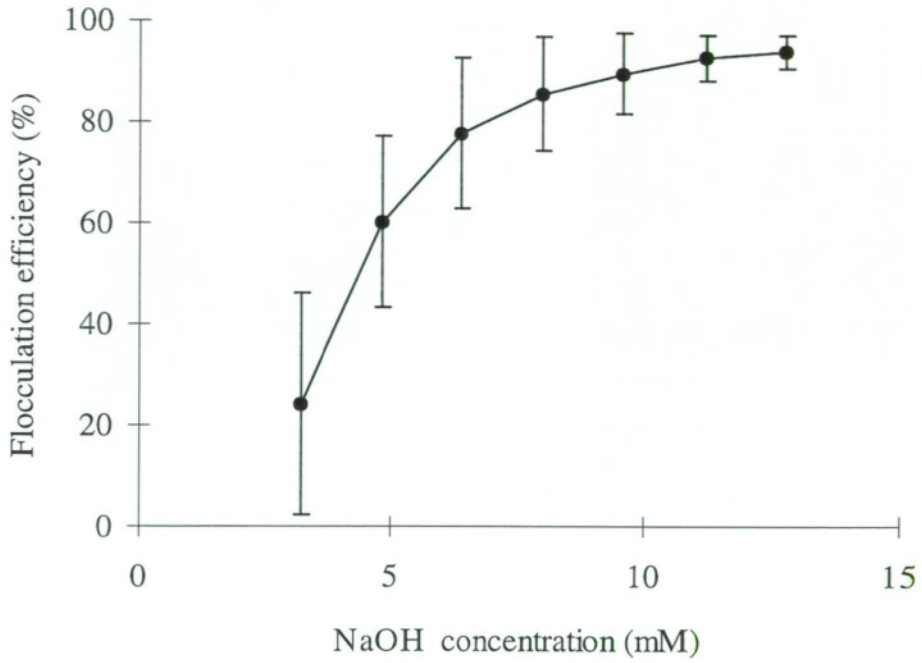


Figure 6.10 Relationship between the rate of NaOH addition (mM) to seawater and the production of flocculated precipitate.

A)



B)

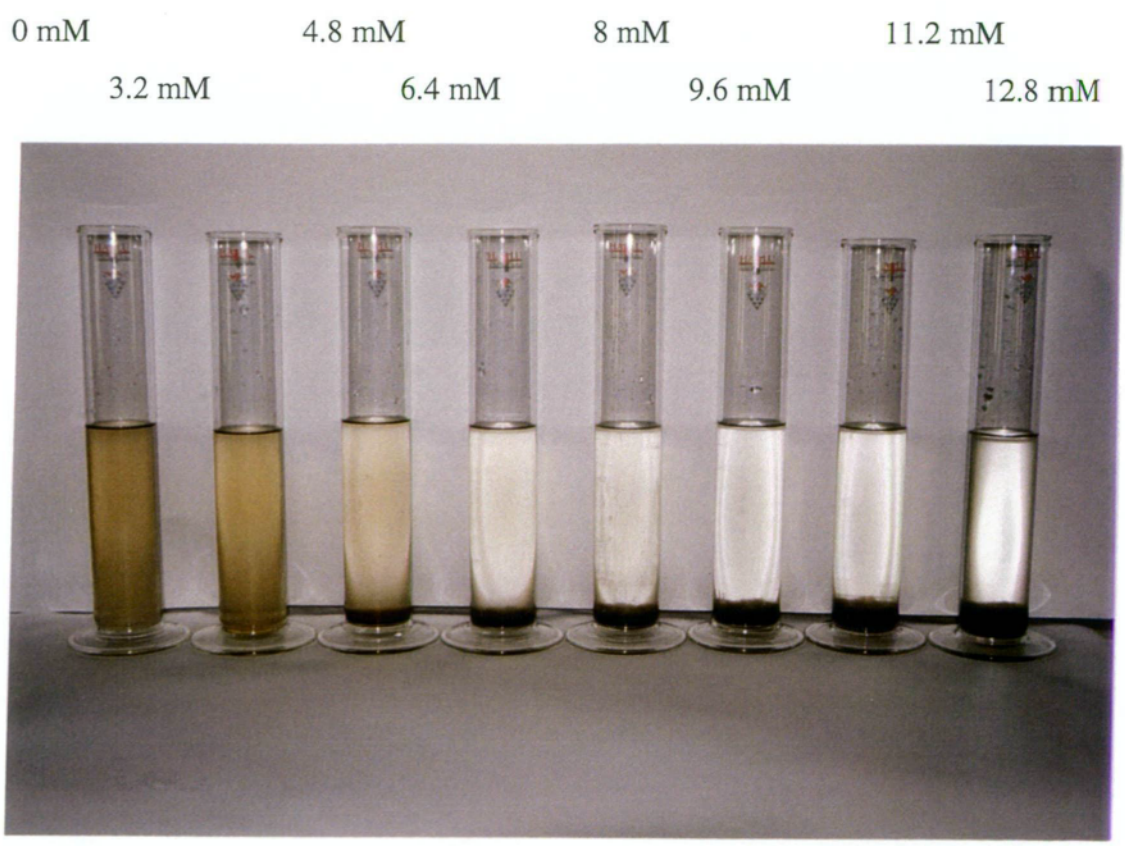


Figure 6.11 A) Flocculation efficiency of *Thalassiosira pseudonana* (250 mL) induced by increasing concentration of NaOH with polyelectrolyte LT-25 (0.05%) added at 1 $mL \cdot L^{-1}$. Error bars are ± 1 S.D. (n=7).
B) Photograph of one replicate taken after 30 min.

Addition of NaOH at 8 mM and polyelectrolyte (0.05% LT-25) at 1 $mL \cdot L^{-1}$ (equivalent to 0.5 $mg \cdot L^{-1}$ algal culture) produced an efficient and rapid flocculation. Adjusting the pH of collected floc significantly reduced the floc volume and increased the overall concentration factor. However, cells were difficult to de-flocculate to single cell status and lower doses of polyelectrolyte were tested to see if cells de-flocculated more readily.

Flocculating *Thalassiosira pseudonana* with NaOH (8 mM) and dosing polyelectrolyte at rates from 0.5-0.0125 $mg \cdot L^{-1}$, showed that rates below 0.5 $mg \cdot L^{-1}$ were still effective (Figure 6.12).

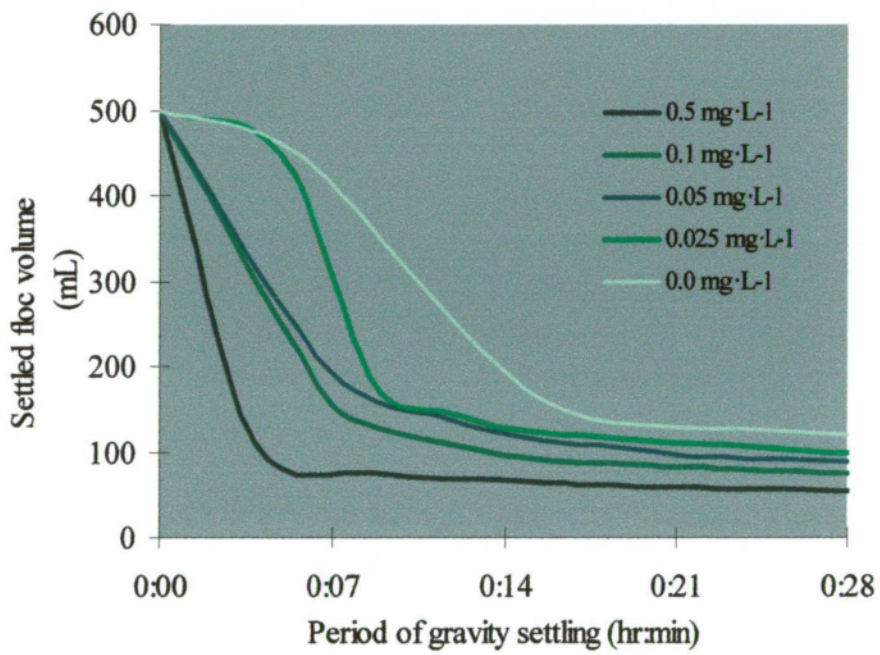


Figure 6.12 Effect of polyelectrolyte concentration on settlement time for floc induced by pH (8 mM NaOH).

Reducing the dose rate of the polyelectrolyte had two effects; 1) flocs were less dense and settled more slowly, 2) the final settled volume of the floc was larger. The dose rate of polyelectrolyte could be reduced 5 to 10 fold without a loss of flocculation efficiency, but with a small increase in settling time and final volume. Adjusting the pH of the collected flocs to pH 7.5-8 resulted in most of the floc dissolving with cell de-flocculating better at polyelectrolyte below 0.5 $mg \cdot L^{-1}$.

6.3 iia. Concentration Factor and Quality of Stored Concentrates

Flocculation experiments were scaled-up to 130 L of algal culture. Three *T. pseudonana* cultures were flocculated by slowly adding NaOH (0.8M) with aeration mixing to a final concentration of 8 mM. Polyelectrolyte (0.05% LT-25) was then added at 1 $mL \cdot L^{-1}$ and flocs allowed to settle. Floc formation was rapid and efficient (89%) and flocs had settled to 5 L by 15 min. The pH of collected floc was then adjusted. In one trial the pH was adjusted to pH 8.7 and floc volume was reduced to 980 mL after 2.5 hr and to 720 mL overnight. The pH was then reduced further to 7.6 and floc volume fell rapidly to 290 mL and then to 160 mL overnight (4°C). This equalled an approximate 800 fold reduction in volume at 89% harvest efficiency. In another trial, pH was reduced in one step to 6.8 and floc volume fell to 285 mL overnight (4°C). This continued to settle further over 2 d to be 175 mL or a 725 fold reduction in volume.

Cell concentrates were divided in half and one half was diluted 1:2 with 1% xanthan gum (in seawater). They were then stored at 4°C in full, sealed tubes. Concentrates without xanthan gum settled with storage and required mixing when sampled but those with xanthan gum (0.5% final concentration) remained as homogeneous suspension, even after prolonged storage (60 d).

Chlorophyll *a* concentration was measured during storage and pigment extracts were scanned to detect shifts in spectra that would indicate the formation of chlorophyll breakdown products (phaeopigments). Chlorophyll levels were much more stable than those measured in centrifuged algal pastes. In two of the three cell concentrates the chlorophyll concentration increased during the first 3 weeks of storage (Figure 6.13 and 6.15). In the remaining concentrate chlorophyll levels decreased with time (Figure 6.14). Scans of chlorophyll extracts from the 3 concentrates showed a slow rate of formation of phaeopigments compared to centrifuged algal pastes. For one *T. pseudonana* concentrate, there had been only a partial shift in the chlorophyll Soret peak from λ430 nm to λ410 nm after 60 d of storage at 4°C (Figure 6.16). The other two pastes had higher λ410 nm peaks but the λ430 nm, chlorophyll *a* peak was still present.

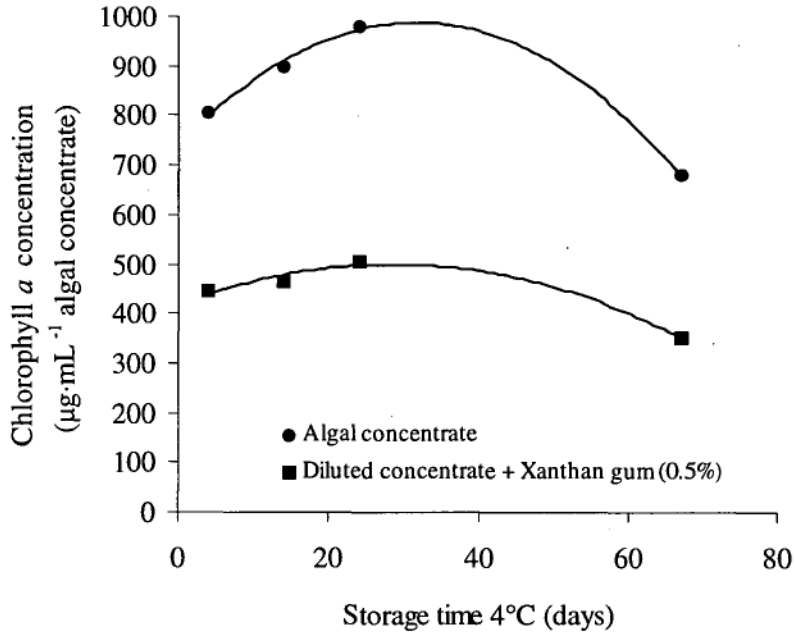


Figure 6.13 Chlorophyll *a* concentration in a pH induced flocculated concentrate of a *Thalassiosira pseudonana* culture (130L). The concentrate was stored at 4°C in sealed, full tubes with and without the addition of 0.5% xanthan gum.

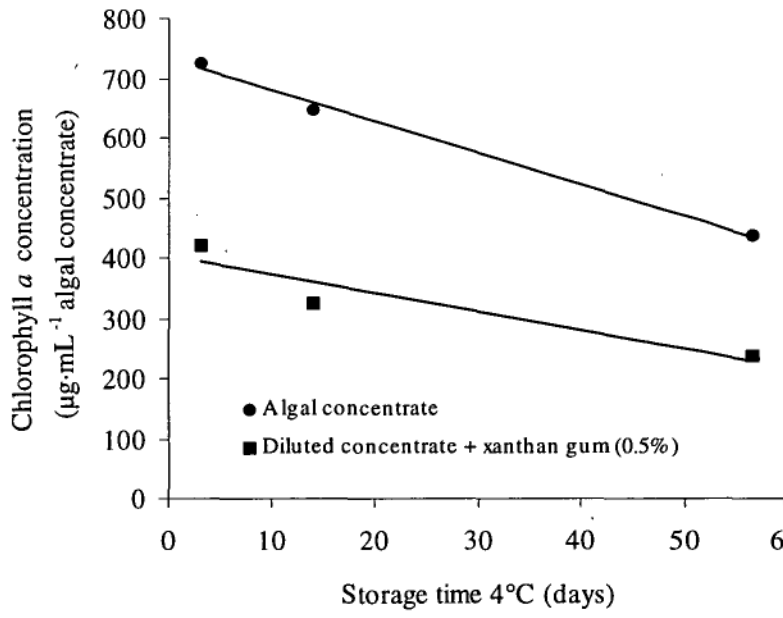


Figure 6.14 Chlorophyll *a* concentration in a pH induced flocculated concentrate of a *Thalassiosira pseudonana* culture (130L). The concentrate was stored at 4°C in sealed, full tubes with and without the addition of 0.5% xanthan gum.

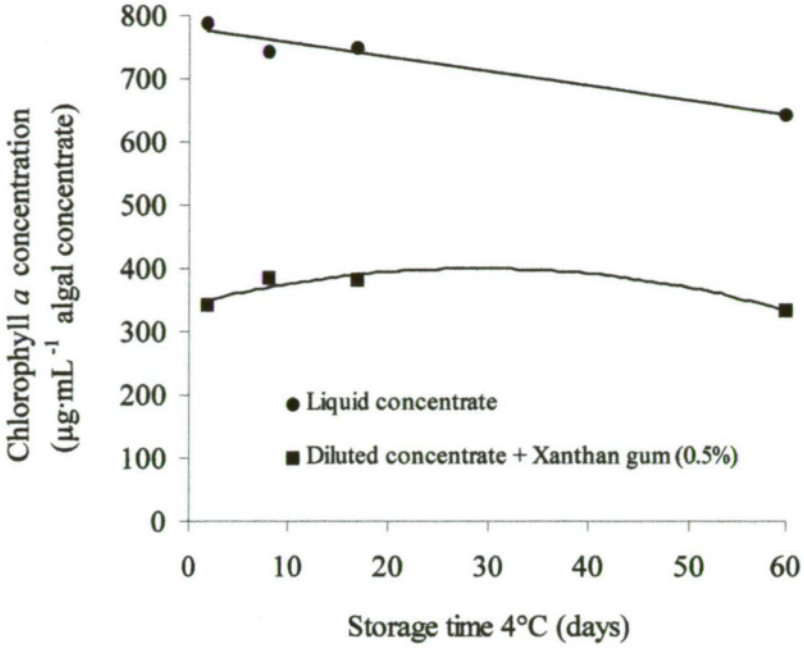


Figure 6.15 Chlorophyll *a* concentration in a pH induced flocculated concentrate of a *Thalassiosira pseudonana* culture (130L). The concentrate was stored at 4°C in sealed, full tubes with and without the addition of 0.5% xanthan gum.

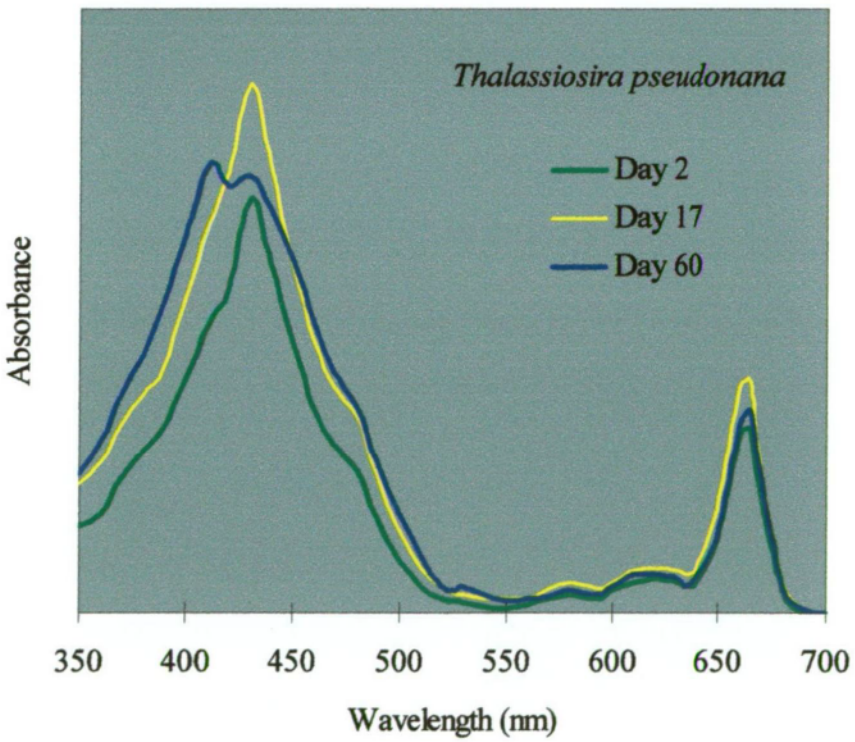


Figure 6.16 Scans of Chlorophyll extracts from a *Thalassiosira pseudonana* concentrate produced by pH induced flocculation and stored at 4°C (without xanthan gum). Chlorophyll concentrations of the concentrate are shown in Figure 6.15.

6.3 iib. Scale-up of pH-Flocculation and Applicability to Other Algal Species

A 500 L culture of *Thalassiosira pseudonana* was pH-flocculated in the same manner as described for 130 L cultures (section 6.3 iia.). Flocculation was rapid (~10 min), efficient (90%) and flocs were larger than those produced in smaller volumes. Flocs were collected and the pH adjusted to pH 7.5. The final concentration factor was 500 fold. There was no indication that the flocculation process could not be scaled-up to larger volumes.

To test the applicability of the pH-flocculation process for other algae, a range of species were flocculated (Table 6.1). Volumes flocculated ranged from 10 L (*Tetraselmis suecica*), to 80 L (*Chaetoceros calcitrans, C. muelleri, Skeletonema* sp., *Rhodomonas salina*), to 130 L (*Attheya septentrionalis*), and up to 500 L for *Nitzschia closterium*. All these species were successfully flocculated with the process occurring as for *T. pseudonana*. The passive nature of the flocculation and deflocculation processes was evident by the high level (40-70%) of motility in cell concentrates of *Tetraselmis suecica* and *Rhodomonas salina*.

Some differences were observed following deflocculation. For the two *Chaetoceros* species, adjustment of pH to 7.5 resulted in complete deflocculation. However, algae were very slow to settle during storage at 4°C, taking 5-6 d for the majority of cells to sink. This slow secondary settling (after initial flocculation settling) was probably due to their setae. This problem was overcome by adjusting the pH of floc to 8-8.5, which left the cells slightly clumped with subsequent rapid settling (24 h). Cells were easily resuspended to single cells by gently hand-mixing.

Chemical analysis was not undertaken for these trials but microscopic examination indicated that all species except *Rhodomonas salina* were in good condition after 4 weeks. Concentrates of *R. salina* were only stable for ~1 week followed by a rapid deterioration and cell lysis. Cultures of *R. salina* have a short stationary phase followed by a rapid decline in cell viability and this is the likely cause of the loss of quality of the cell concentrates.

6.3 iii. Evaluation of Algal Concentrates as Diets for Juvenile Pacific Oysters

6.3 iiia. Gross Composition of Algal Diets

The live *Thalassiosira pseudonana* control diet and algal concentrate (excluding ferric-flocculated diet) diets were fed at a similar daily DW and AFDW of 10.22 ± 0.22 mg (total = 225 ± 5 mg) and 8.33 ± 0.57 mg (total = 183 ± 12 mg) respectively (Table 6.3). The ferric-flocculated diet contained 84% of the Fe^{+3} (as hydroxides) used to induce flocculation, equivalent to 22 mM Fe^{+3} in the diet. This insoluble matter contributed to the high ash content (30% DW) of this diet and it was fed at a daily rate equivalent to 10 mg, calculated from the initial culture dry weight and accounting for the flocculation efficiency and harvest concentration factor.

The protein content (DW) of the diets ranged from 32.1% for the ferric-flocculated diet to 45.7% for the laboratory-centrifuged diet with an average of 39.7% (CV= 12%). Although the ferric-flocculated diet had the lowest percentage, it had the highest total protein fed during the trial (109 mg) due to its higher dose rate, the average total protein fed was 95 mg (CV= 9%). The percentage (DW) lipid of the diets ranged from 26.8% for the ferric-flocculated diet to 45.6% for the live algal diet with an average of 35.8% (CV= 18%). The total lipid fed was lowest in the super-centrifuged diet (70 mg) and highest in the live algal diet (104 mg) with an average of 85.6 mg (CV= 14%). Dietary carbohydrate was the most variable component. The average total carbohydrate was 8.3% DW (CV= 43%) and 19.7 mg (CV= 37%). Much of the variation was due to the low percentage (DW) of mono-oligosaccharides in the Alfa Laval and Super-centrifuged diets (0.85%) compared to the remaining diets (average =3.44%).

Table 6.3 Gross composition and energy content of algal diets fed to *Crassostrea gigas* spat. Diet compositions are shown as total milligrams fed over the duration of the trial and as percentage algal dry weight (% DW).

		Thalassiosira pseudonana diet formulations											
	Unfed control	live algae		pH-flocculated		ferric-flocculated		laboratory centrifuged		Alfa Laval centrifuged		supercentrifuge centrifuged	
Diet gross composition		(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)	(mg)	(% DW)
Protein		87.3	37.9	97.8	43.6	108.7	32.1	99.1	45.7	86.0	37.8	93.1	41.2
Lipid		104.5	45.6	78.3	34.8	90.1	26.8	82.7	38.2	88.1	38.8	69.7	30.8
Polysaccharide		19.0	8.4	15.6	6.9	17.2	5.1	11.0	5.0	10.4	4.6	9.5	4.2
Mono- oligosaccharides		11.2	5.7	8.8	3.8	4.4	1.2	7.2	3.1	2.1	0.9	1.8	0.8
Total carbohydrate		30.2	14.2	24.4	10.7	21.6	6.3	18.2	8.1	12.5	5.4	11.3	5.0
Ash		26.3	11.2	39.8	17.7	100.2	30.0	36.5	16.9	45.6	20.0	59.2	26.2
Total gross components		248	109	240	107	321	96	236	109	232	102	233	103
Total dietary algae (DW)	0	228	100	225	100	334	100	217	100	228	100	226	100
Dietary energy		(J)	(%)	(J)	(%)	(J)	(%)	(J)	(%)	(J)	(%)	(J)	(%)
Energy from protein		1756	27	1965	36	2184	36	1991	35	1729	32	1872	39
Energy from lipid		4148	65	3109	56	3578	58	3283	59	3498	64	2767	57
Energy from carbohydrate		531	8	430	8	380	6	320	6	220	4	199	4
Total dietary energy	0	6434	100	5503	100	6142	100	5594	100	5447	100	4838	100

6.3 iiib. Growth and Composition of Oysters

The percentage increase in oyster DW and AFDW are summarised in Table 6.4 with the average apparent growth efficiency and oyster gross composition data. Over the 22 d experiment, the average instantaneous growth rates of oysters, k (determined from AFDW), were all significantly different except for those fed the ferric-flocced and Alfa Laval centrifuged diets (Figure 6.17).

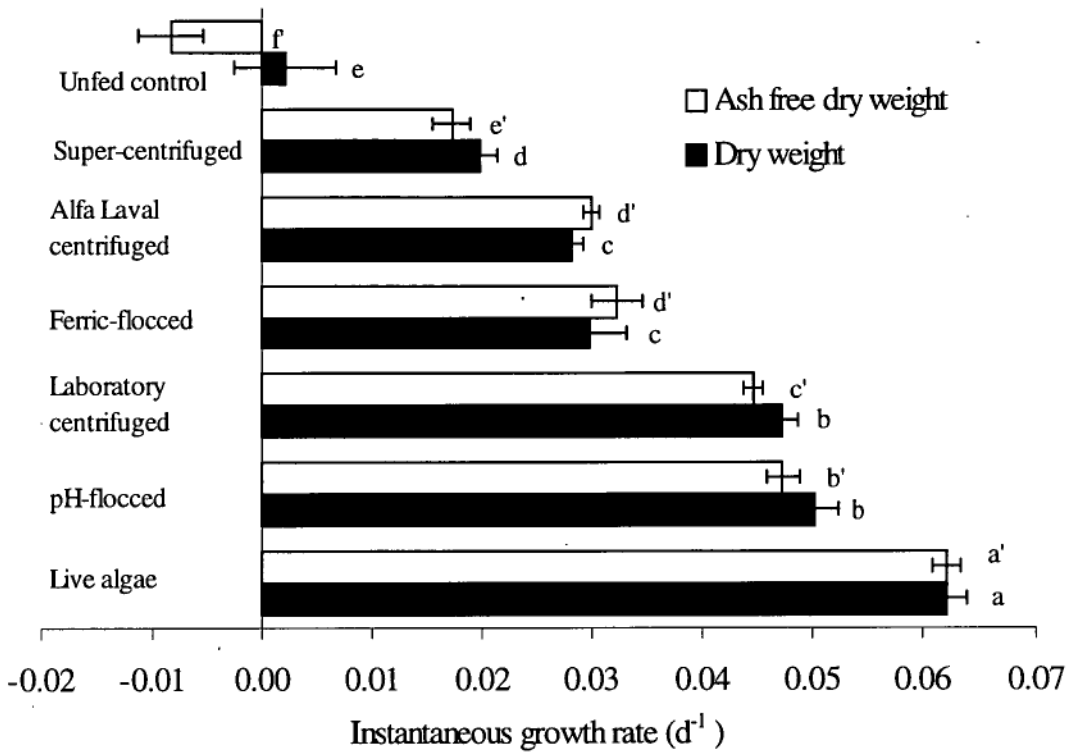


Figure 6.17 Average instantaneous daily growth rate (DW and AFDW) for oysters fed a range of experimental diets. Bars without a common letter are significantly different ($P<0.05$). Error bars are ± 1 S.D. (n=4).

The live algal diet produced the fastest growth rate ($k= 0.062\ d^{-1}$). Of the paste and concentrate diets, the pH flocced diet produced the fastest growth rate ($k= 0.047\ d^{-1}$), just ahead of the laboratory centrifuge diet ($k= 0.044\ d^{-1}$). The super-centrifuged diet produced the lowest growth rate ($k= 0.017\ d^{-1}$), and all diets outperformed the unfed control oysters ($k= -0.008\ d^{-1}$) which lost AFDW. Oyster DW showed similar trends, except oyster growth rates for pH-flocced and laboratory centrifuged diets were not significantly different, also the unfed oysters showed a small weight gain.

The average instantaneous growth rates show only part of the diet-effects on oyster growth. Weekly measurements of oyster AFDW and DW revealed much more about the nutritional value of the diets (Figure 6.18). From these, instantaneous daily growth rates (AFDW d^{-1}) during each week of the feeding experiment were calculated (Figure 6.19).

Table 6.4 Gross composition (% AFDW), growth and apparent growth efficiency (%) of juvenile oysters fed a range of algal diets. Error values are ± 1 S.D. (n=4).

	initial oysters	unfed control	live algae	pH-flocced	ferric-flocced	laboratory	Alfa Laval	Supercentrifuge
Oyster gross composition (% AFDW)								
Protein	43.7 ±2.59	51.8 ±4.09	44.3 ±2.25	50.9 ±2.48	48.7 ±3.12	48.6 ±2.18	46.3 ±1.54	51.9 ±4.07
Lipid	14.9 ±1.26	10.8 ±0.64	16.1 ±1.16	11.7 ±0.40	14.7 ±1.21	14.2 ±0.17	18.2 ±1.08	12.2 ±1.55
Polysaccharide	5.3 ±0.45	5.0 ±0.41	4.0 ±0.32	4.1 ±0.20	4.1 ±0.20	4.0 ±0.13	4.0 ±0.07	5.0 ±0.29
Mono- oligosaccharides	2.5 ±0.14	1.4 ±0.09	4.9 ±0.87	1.9 ±0.19	2.0 ±0.26	2.0 ±0.27	1.9 ±0.50	1.6 ±0.06
Total carbohydrate	7.8 ±0.45	6.4 ±0.47	9.0 ±1.17	6.0 ±0.14	6.2 ±0.31	5.9 ±0.39	5.8 ±0.47	6.6 ±0.32
Total gross components	66	63	69	69	70	69	70	71
Oysters								
DW (mg ·oyster^{-1})	1.22 ±0.03	1.29 ±0.14	4.82 ±0.19	3.70 ±0.16	2.36 ±0.16	3.46 ±0.12	2.28 ±0.05	1.90 ±0.06
AFDW (µg ·oyster^{-1})	93 ±4.26	77 ±5	364 ±10	263 ±8	189 ±9	247 ±5	179 ±3	136 ±5
Percentage increase in oyster DW		5 ±11	293 ±15	202 ±13	93 ±14	183 ±10	86 ±4	55 ±5
Percentage increase in oyster AFDW		-16 ±6	292 ±11	184 ±9	104 ±10	167 ±5	93 ±3	46 ±6
Total diet AFDW (mg)		0	202	185	234	181	182	167
Total increase in oyster AFDW (mg)		-5	85	55	31	51	29	15
Average apparent growth efficiency (%)			42	30	13	28	16	9

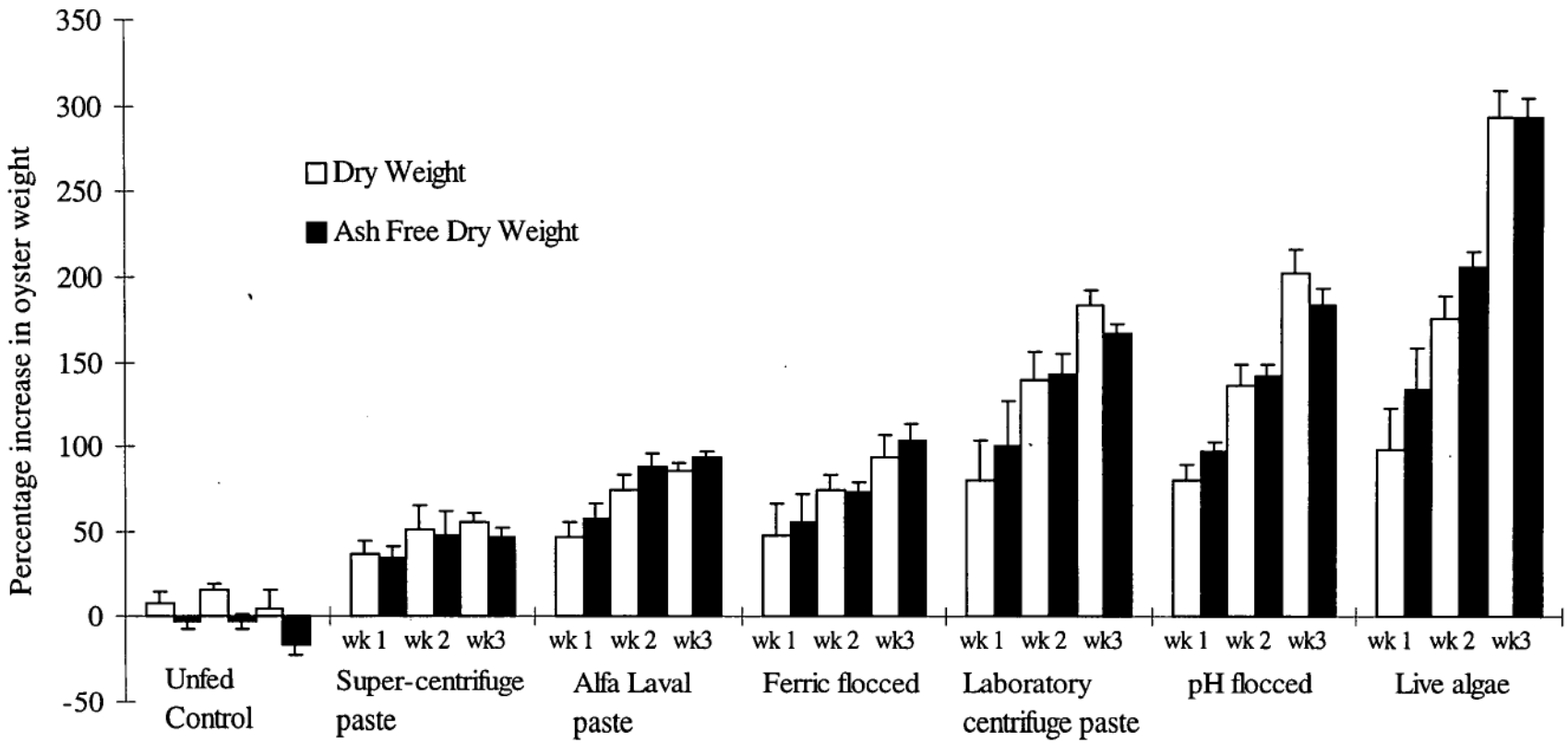


Figure 6.18 Oyster growth measured as the percentage increase in DW and AFDW after 1, 2 and 3 weeks of feeding on experimental diets of *Thalassiosira pseudonana*. Error bars are ± 1 S.D. (n=4).

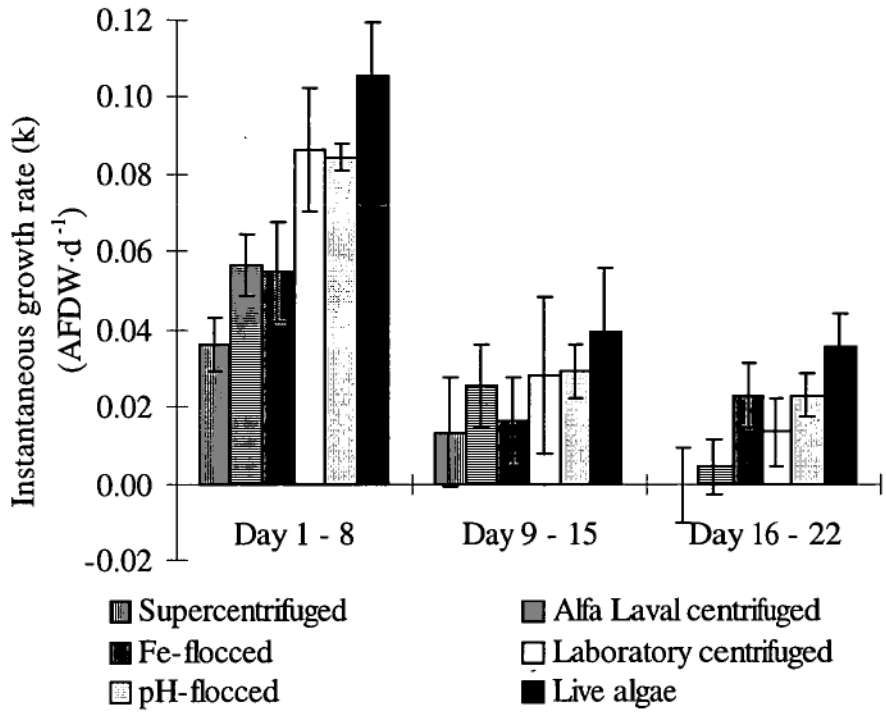


Figure 6.19 The average instantaneous growth rate for oysters fed each diet during day 1-8, day 9-15 and day 16-22. Error bars are ± 1 S.D. (n=4).

The most noticeable result was that the growth rate of all diets declined significantly from the period day 1-8 to that during day 9-15. This occurred because the ration delivered per bucket was kept constant throughout, yet oyster biomass increased significantly (Table 6.5). The day 1 feed rate of all diets, except ferric-flocculated, was 0.30 mg algae·mg oyster^{-1} and the rate at which it fell was dependent on the increase in oyster AFDW. The average feed rates during the first 8 days were lowest for the fastest growing treatments; live algal, laboratory centrifuged and pH-flocced diets. At the end of each week, fifty oysters were removed for measurement of growth, this partially re-set the feeding rate between periods. Because of this, the average daily feed rates from day 9-15 and day 16-22 were almost identical as the increase in oyster AFDW was matched by removal of the 50 oyster sample.

Table 6.5 The average daily algal feed rate for each diet replicate during the periods; day 1 - 8, day 9 - 15 and day 16 - 22.

Diet	Day 1 - 8	Day 9 - 15	Day 16 - 22
	Feed rate AFDW (mg algae·mg oyster $^{-1}$)		
Unfed control	0	0	0
Super-centrifuged	0.193	0.192	0.221
Alfa Laval centrifuged	0.193	0.167	0.189
Ferric-flocced	0.316	0.175	0.224
Laboratory centrifuged	0.168	0.131	0.131
pH-flocced	0.171	0.133	0.138
Live algae	0.164	0.121	0.112

The feed rate of the super-centrifuged and Alfa Laval-centrifuged diets were quite constant over the experimental period because of the relatively low level of oyster growth that they supported. However, the average daily growth rate fell each week, as the oysters became malnourished. Oysters fed the super-centrifuged diet had a negative daily growth rate (k= -0.0003 d^{-1}) from day 16-22 as oysters lost AFDW, while oysters fed the Alfa Laval-centrifuged diet grew slowly (k= 0.045 d^{-1}) during the same period.

From calculations of the algae fed (mg AFDW·period^{-1}) and the increase in oyster weight (mg AFDW·period^{-1}), the apparent growth efficiency for each period was calculated (section 4.2 i.). The difference between apparent growth efficiency and daily growth rates is that it compensates for feed ration. This was most noticeable for the ferric-flocced diet that was unintentionally fed at a higher rate than the other diets. The graph clearly shows the effect of a fall in the weight-specific ration and also the rate of loss of nutritional value of the different diets.

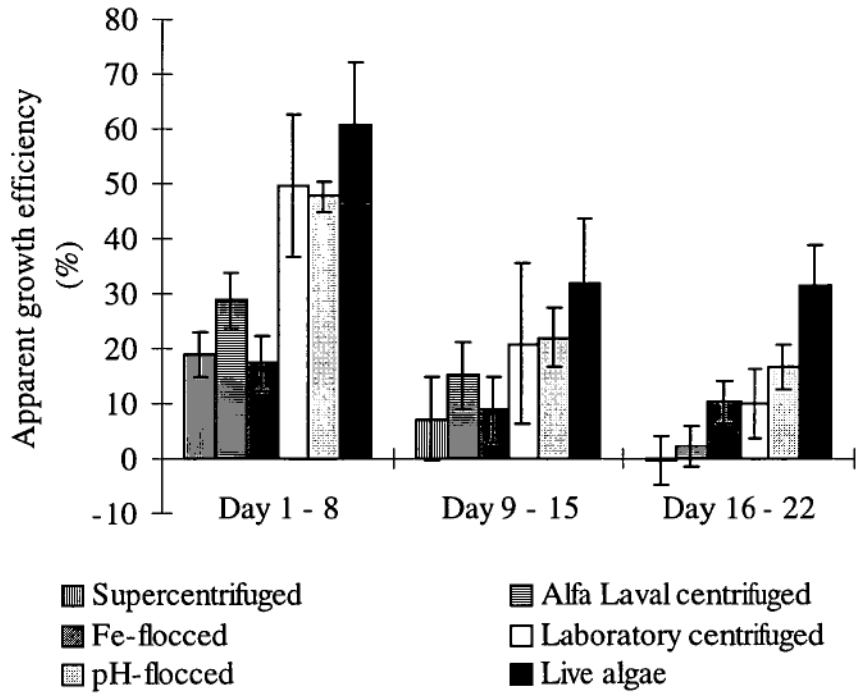


Figure 6.19 The average weekly apparent growth efficiency for oysters fed each diet during day 1-8, day 9-15 and day 16-22. Error bars are ± 1 S.D. (n=4).

6.4 *Discussion*

Flocculation was an efficient (>85%) method to concentrate a range of microalgae used as aquaculture feeds. This compares well to reported efficiencies of over 90% for ferric chloride flocculation of marine algae (Sukenik *et al.*, 1988), and ≥75% for chitosan flocculation (Lubián, 1989). It is a rapid and relatively simple method that does not require the purchase of expensive machinery, as for methods involving centrifugation. Another advantage of flocculation is that it essentially a volume-independent process limited only by the size of the culture vessel, whereas centrifugation is a rate-limited process. To increase harvest rate by centrifugation can require the purchase of a larger, expensive machine.

Algal concentrates produced by flocculation were shown to be nutritious diets for juvenile Pacific oysters. Although live algae were still a superior diet, the pH-flocculated diet produced an instantaneous growth rate (AFDW) equal to 71% of that of the live algal diet. This was significantly greater growth than that from diets produced by centrifugation, in the same feeding experiment and in those discussed in Chapter 5.

Flocculation of marine microalgae using the coagulant $FeCl_3 \cdot 6H_2O$, requires concentrations 5 to 10 times higher than those required for freshwater microalgae (Sukenik et al., 1988). This is due to the high ionic strength of the media reducing flocculant chemical activity and masking its active sites. For *Thalassiosira pseudonana,* flocculation was most rapid and efficient (>90%) at Fe^{+3} concentrations above 500 μM. This produced large flocs that were resistant to disruption and settled rapidly. However, the density of algae within the floc was much lower (~10 fold) than flocs produced at Fe^{+3} concentrations below 250 μM. Pre-adjustment of culture pH improved flocculation efficiency with an initial optimal pH of 6. This in agreement with Alaerts and Van-Haute (1981), who report pH 6 to be optimal for flocculation with ferric chloride or alum. However, flocculation efficiency increased further at pH levels above 9 where the Fe^{+3} concentration could be reduced to 125 μM with 80% flocculation of algal cells.

Increasing the pH of seawater above 10 resulted in formation of precipitate that continued to develop with increasing addition of OH^-. Millamena *et al.* (1990), also observed precipitation when culture pH was raised to pH 10. Although they postulated that this was due to the precipitation of organic ions and excess nutrients, it is likely to have been due to the precipitation of Ca and Mg hydroxides since precipitate forms in clean oceanic water. In this study, NaOH was used to increase pH, but a similar result can be obtained using lime (Sripayatt, 1982; Millamena et al., 1990a). Subsequent addition of the non-ionic polyelectrolyte LT-25, caused flocculation of the precipitate, forming white flocs. When performed on algal cultures, cells are trapped in the floc which settles, concentrating the algae. Minimal bulk of floc with maximum concentration of cells was produced close to the point of formation of visible precipitate, 6-10 mM NaOH. Additional NaOH did not improve efficiency of algal flocculation, but resulted in increased bulk of precipitate, which formed loose flocs that did not pack so tightly when settled.

Flocculated algae have been used as diets for aquaculture species. Sandbank (1978), fed microalgae grown in waste-water and flocculated with aluminium sulfate to common carp (*Cyprinus carpia*). A diet containing 25% algal meal produced growth comparable to the control diet with no harmful effect of the algae or aluminium on the growth rate or health of the fish. Millamena *et al.* (1990a), fed *Penaeus monodon* larvae dried, flocculated diets of *Chaetoceros calcitrans* and *Tetraselmis chuii.* However, to use flocculated algae as diets for oysters they must be deflocculated. Flocs produced using Fe^{+3} or NaOH settled rapidly (<15 min), where they were collected and de-flocculated to release the algal cells. Flocs produced using Fe^{+3} were successfully de-flocculated using the chelating agent EDTA. It was added to the floc at pH 4.5 on an equal molar ratio to the measured iron concentration. Cells were separated from the iron solution by centrifugation and resuspension in fresh seawater. Although this method produced a concentrate of mostly single cells, EDTA is likely to result in damage to cell membranes and leakage of metabolites. EDTA is used to extract periplasmic proteins from bacteria where it releases a large fraction of the lipopolysaccharides and makes the outer membrane more permeable with loss of osmolytes (Leive and Davis, 1980). The low pH also resulted in conversion of chlorophyll to phaeopigments. Because of possible

loss of quality of the cells, concentrates produced by Fe^{+3} flocculation and used in feeding experiments were not de-flocculated. They were produced using low Fe^{+3} concentrations and partially resuspended by rapid shaking before feeding.

Flocs produced by addition of NaOH were successfully de-flocculated to single cells by adjustment of pH to 7.5 to 8 using HCl. During this procedure the bulk of the floc dissolved and the cells formed small flocs that consisted almost entirely of algal cells. Re-flocculation of these cells to further concentrate them was unsuccessful and is likely a result of the altered chemistry of the water and the higher algal load. However, it was not required since the small flocs settled on standing (24-48 hr) and the concentration factor increased from an initial ~25 fold to 700-800 fold. These concentrated flocs were easily dispersed with gentle shaking and dilution for feeding to juvenile oysters.

As discussed in chapter 1 (section 1.4), the flocculation of freshwater microalgae, can be achieved using polymers (chitosan, and polyelectrolyte compounds derived from polyacrylamide) as the sole flocculating agent, but salinity levels above 5 $g \cdot L^{-1}$ inhibits flocculation. Therefore, in marine systems polymer flocculants are often used in conjunction with inorganic coagulants (Fe^{+3}, alum, lime) where they improve flocculation efficiency. In this study, the polymer LT-25 (non-ionic polyacrylamide derivative) was routinely used at 0.5 $mg \cdot L^{-1}$, but experiments showed that this level could be reduced 5 to 10 fold without a loss of flocculation efficiency. There is little data published on the ecological or toxicological effect of synthetic polymer flocculants. Beim and Beim (1994) found polymer flocculants negatively affected all water ecosystems and the most acute effects were from exposure to cationic polymer flocculants. They recommended that maximum permissible concentrations be set for flocculant residues. The non-ionic polymer LT-25 is a low toxicity polymer manufactured for the clarification of drinking water and with residual non-polymerised acrylamide below permissible levels. In this study it was not possible to determine the residual quantity of polymer in the final cell concentrate. However, assuming all added polymer (0.5 mg LT-25$\cdot L^{-1}$) remained in the cell concentrate (700 fold final concentration factor) then the maximum concentration would be 0.35 $mg \cdot mL^{-1}$. Cell concentrates were then diluted ~1:5000 for feeding to juvenile oysters. There was no

increase in mortality observed in oysters fed flocculated concentrates and the pH-flocculated diet outperformed even the most gently centrifuged algal diet.

Flocculated algal cells, although densely packed in flocs, were indistinguishable from non-flocculated cells when viewed by light microscopy. This contrasts with centrifuged algal cells that were clearly damaged, except for some small, tough green algal species as discussed in Chapter 5. For pH-flocculates, de-flocculation produced dense, single cell suspensions of cells that appeared unaffected. For motile species such as *Tetraselmis suecica* and *Rhodomonas salina*, between 40-70% of cells were still motile, indicating very little disruption to cells. This demonstrates the low-shear nature of flocculation compared to continuous-flow centrifugation where concentration of another cryptophyte *Geminigera cryophila*, produced an algal paste consisting entirely of cell fragments (Chapter 5.3 i.).

Chlorophyll *a* measurements also indicated that cell concentrates produced by flocculation were in better physical condition than those produced by centrifugation. Spectrophotometric scans of chlorophyll extracts of 21 d old, pH-flocculated concentrates of *Thalassiosira pseudonana*, showed no shifts in absorbance peaks that would indicate degradation of chlorophyll and formation of phaeopigments. This compares to centrifuged pastes of *T. pseudonana*, where by day 24, scans of chlorophyll extracts showed only peaks characteristic of phaeopigments (Figure 5.13a & c). After 50 to 60 d of storage at 4°C, scans indicated a mixture of chlorophyll *a* and phaeopigments similar to a 10 d old centrifuged paste (Figure 6.16 and 5.13a).

Algal concentrates (pastes) produced by centrifugation using an Alfa Laval cream separator or Sharples Supercentrifuge, had a DW of 128 $g \cdot L^{-1}$ and 147 $g \cdot L^{-1}$ respectively. Those produced by flocculation or laboratory centrifuge were liquid concentrates with a DW of 6 to 7 $g \cdot L^{-1}$. Tredici *et al.* (1996), found that cell concentration ($g \cdot L^{-1}$) was a highly significant factor in determining the level of viability (motility and photosynthetic activity) of algal cells stored (4°C) as concentrates. For centrifuged concentrates of *Tetraselmis suecica* at 4 $g \cdot L^{-1}$ cell concentration, they found no significant reduction in cell viability during the first 50 days of storage at 4°C. At

cell concentration of 20 and 60 $g \cdot L^{-1}$, a high level of viability (>70%) was maintained until around 10 days, then it rapidly declined to total mortality by 21-24 days. For algal pastes, cell viability rapidly declined after 4 days to be nil after two weeks. The flocculated and laboratory centrifuged cell concentrates at 6-7 $g \cdot L^{-1}$ DW, are likely to be within a concentration range appropriate for maintaining a quality product. This may partly explain their higher nutritional value to juvenile Pacific oysters. However, cells in pastes of *T. suecica* produced using the Alfa Laval cream separator retained no level of motility after resuspension (Figure 5.6), indicating the machine is more damaging than that used by Tredici *et al.* The poor nutritional value of algal pastes used in this study may in part be due to their cell concentration, but damage to cells during centrifugation and subsequent leakage of cell metabolites is likely to have been the determining factor.

During the feeding experiment, measurement of weekly growth of oysters provided more insight into the nutritional value of diets than a single final measurement, as in feeding experiments discussed in Chapters 4 and 5. Firstly, they demonstrated the effect on growth rate of a falling weight-specific feed ration, resulting from oyster growth. If "scope for growth" is defined as the energy of the assimilated ration available for somatic and/or germinal tissue growth, once metabolic energy requirements have been met (Warren and Davis, 1967), then the instantaneous growth rate and apparent growth efficiency will fall as the weight-specific feed ration falls. This was seen for oysters fed the live algal control diet where the instantaneous growth rate and apparent growth efficiency (AFDW) fell from $0.105 \cdot d^{-1}$ (61%), during the first eight days to $0.039 \cdot d^{-1}$ (32%), during the following seven days. This was due to a 26% fall in the average daily feed rate because of growth of oysters. The weekly feed rate of oysters fed algal pastes (Alfa Laval and Supercentrifuge) were almost constant, as any increase in overall oyster mass was balanced by removal of oysters for measurement of weekly growth.

Secondly, weekly growth measurements showed the loss of nutritional value of diets with prolonged feeding. They showed that oysters fed the supercentrifuged algal paste failed grow after two weeks of feeding and on average, oysters lost organic weight during the third week. The Alfa Laval paste was only marginally better with an

instantaneous growth rate during the third week of only $0.004 \cdot d^{-1}$. The rate of decline in growth indicated growth would have stopped had the experiment continued to four weeks. Weekly apparent growth efficiencies, are a measurement of the increase in oyster tissue AFDW relative to the level of dietary AFDW and partly compensate for differences in feed rate that occur due to differential oyster growth. The main difference between weekly apparent growth efficiency measurements and instantaneous growth was for results of the ferric-flocced diet. This diet was unintentionally fed at a higher rate than other diets due to an underestimation of the AFDW relative to DW, which included the inorganic floc precipitate. Apparent growth efficiency measurements compensated for this difference.

The average instantaneous growth rates (AFDW) over the experiment showed that oysters fed the pH-flocced diet grew significantly faster than all other diets except the live algal control (Figure 6.17). However, growth in oysters fed the laboratory centrifuged and pH-flocced diets were not significantly different during the first two weeks. Weekly instantaneous growth and apparent growth efficiency measurements showed that the nutritional value of both diets declined from week 2 to week 3. This was evident from less growth even though the weight-specific ration remained unchanged during this period. The pH-flocced diet became significantly better during the third week because its nutritional value deteriorated more slowly than that of the laboratory centrifuged diet. The ferric-flocculated diet also appeared to lose nutritional value slowly, but growth was less than either the pH-flocced or laboratory centrifuged diet. This was likely to have been due to only limited de-flocculation of cells, leaving an unknown portion of the diet at a size too large for filtration by the oysters.

Final biochemical analysis of oysters showed no clear trends in relation to the gross composition of diets and oysters. In Chapter 4, oysters composition was found to be correlated with dietary protein and carbohydrate when fed mixed diets of live algae. For those diets, oyster growth was not affected by nutritional imbalances and deficiency as occurred in oysters fed some algal concentrate diets.

Flocculation and de-flocculation by pH is a simpler and more effective method than ferric flocculation for producing nutritional algal concentrates suitable for filter feeding species. It has been shown to be an efficient method for the concentration of diatoms, prasinophytes and cryptophytes. Concentrates produced by pH-flocculation are of an initial nutritional value similar to those produced by laboratory-scale centrifugation but have better storage capacity. Laboratory centrifugation (non-continuous, low x g) is a relatively gentle centrifugation method and would produce one of the best possible centrifuged concentrates. However, it is not a practical centrifuge method to scale-up and this process still adversely affected algal cells. Flynn and Al-Amoudi (1988) found that *Phaeodactylum tricornutum* cells lost a significant portion of their intracellular amino acids when centrifuged in a laboratory centrifuge (4000 x g). The more rapid loss of nutritional value of the laboratory centrifuged algal concentrate compared to the pH-flocculated algal concentrate is not a result of differences in storage conditions. As already discussed, increasing cell density of concentrates adversely affects their condition when stored, but both concentrates were of a similar DW density. The more rapid loss of nutritional value may be a result of low level cell damage during centrifugation.

Chapter Seven

General Discussion

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7.1. *Introduction*

This thesis has focused on the isolation, characterisation and evaluation of Australian microalgae as feed species for juvenile Pacific oysters (*Crassostrea gigas*) and in the formulation and evaluation of microalgal concentrates as an alternative to the use of live algae cultures in the aquaculture industry. In the evaluation of Australian microalgae their gross and biochemical composition were used to select suitable test species and to explain the differences in their performance as aquaculture feeds. These results show nutritional details that are significant not only for the tested algal species but also for other algal isolates and oyster diets in general. Methods developed to produce microalgal concentrates have application with many traditional feed species and also with the isolated Australian species. The developed methods and the nutritional evaluation of algal concentrates have provided significant new information on the use of alternatives to live algal cultures for aquaculture feeds. The results complement recent research in this field and have commercial application. The significance of specific findings are considered in detail at the end of each chapter. In concluding this work, I would like to focus on the major findings, their significance and highlight additional areas worthy of investigation.

7.2. Aquaculture Potential of Australian Microalgal Isolates

Two Australian diatom isolates *Attheya septentrionalis* and *Entomoneis* cf. *punctulata* were highly nutritious feed species for juvenile Pacific oysters. No citations in the literature were found in reference to the use of these species as aquaculture feeds. Now as nutritionally evaluated microalgae, they can be included in the collection of other evaluated species available to the aquaculture industry. Results of the feeding experiment to evaluate four Australian isolates (ie. *Attheya septentrionalis, Entomoneis* cf. *punctulata, Extubocellulus spinifera* and *Thalassiosira oceanica*), showed that protein level of a mixed algal diet was the single most significant factor in determining a nutritious diet for the tested oysters assuming that all lipid requirements were satisfied.

Nitzschia cf *paleacea* (CS-429) and *Minidiscus trioculatus* (CS-435) were two isolates that grew rapidly (1.4 divisions·d^{-1}) and had a high protein level (41% DW) in stationary phase. These isolates could also have potential as feed species for juvenile oysters. *Nitzschia* cf *paleacea* (CS-429 and CS-430) could both have application as feed species for juvenile abalone where *Nitzschia closterium* is often used. The chlorophyte isolate (*Chlorella*-like CS-436) was a species that when concentrated by centrifugation to a paste, could be stored for extended periods. As a paste, this species could find use in the intensive culture of marine rotifers. Yoshimura *et al.* (1996) developed an intensive rotifer cultivation process based on the use of algal pastes of freshwater *Chlorella* that was 100 times more efficient than conventional methods.

The isolation and identification of Australian microalgae has also added to knowledge on the global distribution of microalgae. *Attheya septentrionalis* is described as a polar species. The known distribution of *Extubocellulus spinifera* includes coastal waters of Mexico, Germany, North America and Australia (Hallegraeff and Burford, 1996). *Papiliocellulus simplex* was only recently described from England (Gardner and Crawford, 1992) and an Australian isolate from the Gulf of Carpentaria (GOC-42, culture now lost) was only the second published report of this benthic species (Hallegraeff and Burford, 1996). *Thalassiosira oceanica* has been observed at inshore and offshore stations in the East Australian Current and on the North West Shelf of Australia (Hallegraeff, 1984). The Australian isolates reflect the homogeneity of many microalgal species throughout the world's oceans.

7.3. Algal Diet Composition and Nutritional Value to Juvenile Pacific Oysters

Gross composition of live mixed-algal diets was found to significantly affect both the growth rate and gross composition of juvenile oysters. Total dietary protein level was the single most significant component in determining oyster growth with dietary energy was the best alternative variable. Oyster growth was not significantly correlated with total dietary levels of carbohydrate or lipid. Increasing levels of dietary protein and carbohydrate were correlated with increasing (% AFDW) levels in oysters at the expense of tissue lipid. Oysters also retained more of the dietary protein (component retention index; CRI(protein)= 0.6-0.88) than either carbohydrate or lipid where very

low retention indices reflected the primary use of these components for metabolic energy. Oysters had a low requirement of the essential PUFA 22:6n-3 and growth did not appear to be affected at a feed composition of ~0.14 to 0.29 % DW.

Although Thompson *et al.* (1993) found no apparent relationship between gross proximate composition of phytoplankton and their nutritional value to *C. gigas* larvae, many other authors have and relationships found in this work support many of those found by other researchers. Webb and Chu (1983) found that the nutritional value of algal diets for oyster was correlated with total protein as opposed to total lipid or carbohydrate. Langton *et al.* (1977) also found dietary protein content to be positively correlated with growth of juvenile Manila clams (*Tapes japonica*). Settlement of larval *C. gigas* was also improved with diets containing higher protein levels (Utting, 1986). Langdon and Waldock (1981) found that growth of *C. gigas* spat was not correlated with the total lipid content of dietary algae and increasing the lipid content of the diet with triolein capsules did not enhance growth of spat.

Algae that ranked highly as diets for *Ostrea edulis* contained high levels of carbohydrate and the essential PUFAs, 20:5n-3 and 22:6n-3, with protein content less critical (Enright *et al.*, 1986a). However, although oyster growth was greater in carbohydrate-rich diets it was found to be of no benefit to oysters if insufficient protein was available (Enright *et al.*, 1986b). Surplus carbohydrate could only be utilised if there was adequate dietary protein. Kreeger and Langdon (1993) fed juvenile mussels (*Mytilus trossulus*) diets enriched with protein microcapsules. They found that the positive growth response of mussels fed these diets was due to increased dietary protein and not simply due to increased total energy. They also found that mussels fed diets rich in protein tended to have more protein and less lipid and carbohydrate in their tissue. Laing *et al.* (1990) also found diet composition to be reflected in the composition of larvae of Manila clam (*Tapes philippinarum*). Higher total carbohydrate content of diets resulted in higher levels in larvae. These results are in agreement with the positive correlation found in this study between higher dietary protein and carbohydrate levels with those of oyster tissue. However, they do not support the findings of Laing and Verdugo (1991) who

found the biochemical composition of juvenile bivalves largely reflected growth rate and not diet composition.

Although the PUFAs 20:5n-3 and 22:6n-3 are essential for *C. gigas* (Langdon and Waldock, 1981), only a low requirement of 22:6n-3 was found in this study and supports findings of Thompson *et al.*, (1993) who found levels between 0.5 to 2% of total fatty acids to be sufficient.

Until a formulated diet is developed for filter feeding bivalves, dietary requirements of aquaculture species will continue to be estimated from the use of variable algal diets of partially controlled composition. These present significant problems in being able to make finite statements on nutritional requirements. In Chapter 1, the large inter-species variation in composition of microalgae was discussed and also how culture conditions influence algal composition. These variations complicate comparisons between published research. Determining relationships between the gross composition of mono-algal diets and oyster growth rate requires careful consideration of essential components (PUFAs, essential amino acids, vitamins), and numerous non-nutritional factors such as cell size, digestibility, toxicity.

The experimental design developed in this study to evaluate Australian isolates used ternary algal diets, which reduced the likelihood of growth limitations caused by deficiency in essential nutrients. The algal class composition of the ternary diets was fixed which further reduced diet-specific factors so that differences in oyster growth were more likely to be due to differences in the gross composition of the tested diets. A deficiency in one of the test diatoms (lack of essential PUFA 22:6n-3) was compensated for by inclusion of the minor prymnesiophyte diet component. The significant negative correlation between percentage of 20:5n-3 in diets and the growth of oysters found by Thompson *et al.*, (1993) was also avoided in this experimental design, as all test diatoms had similar levels of the PUFA. The experimental method was able to evaluate new species, explain differences in diet performance and provided insight into the nutritional condition of oysters. As discussed in Chapter 4, the changes in gross composition of the oysters and the highly conserved nature of oyster protein

in the starved oysters indicated that oyster spat obtained from the nursery may have been protein-limited. If so, this could partly explain the correlation between dietary protein and oyster growth. Kreeger and Langdon (1993) also found growth to be positively correlated to dietary protein and that the sampled juvenile mussels may have been protein limited. Any such nutritional deficiencies in pre-experimental animals may contribute to different nutritional relationships reported by authors for short-term nutritional studies.

7.4. Potential Use of Algal Concentrates as Aquaculture Feeds

Centrifugation was successfully used to produce algal pastes. Green algal species withstood the pasting and storage process the best and included the prasinophyte *Tetraselmis suecica*, and small chlorophyte species. However, more nutritious aquaculture species were damaged by centrifugation and deteriorated with storage. Feeding experiments with juvenile Pacific oysters showed that algal pastes of *Thalassiosira pseudonana* were poor diets and oyster growth ceased after 2-3 weeks of feeding. The high shear forces during centrifugation are the probable cause of cell damage and damaged cells deteriorated with storage and leached organic content on resuspension.

Flocculation was demonstrated to be an efficient method to concentrate microalgae including many that were damaged by centrifugation. The process was gentle and algal cells appeared unaffected by flocculation and deflocculation. Two flocculation methods were developed based on ferric and pH-induced coagulation with the addition of a polyelectrolyte flocculant. Both methods were efficient but pH-flocculation was a more simple process that produced flocs that were much easier to deflocculated by simple pH-adjustment. No problems were encountered in scaling-up flocculation from laboratory scale cultures to 500 L mass culture volumes. Algal concentrates produced by flocculation were shown to be significantly more nutritious to juvenile oysters than centrifuged algae. Diets produced by pH-flocculation were the best of all tested algal concentrates.

As discussed in Chapter 6, the type of centrifuge used to produce algal pastes is likely to be highly significant in relation to the quality of the algal product. Nell and O'Connor (1991) have found centrifuge produced algal pastes to be of greater nutritional value to oysters than those produced by the cream separator used in this study. However, even gentle laboratory centrifugation was shown to produce algal concentrates that deteriorated more quickly than those produced by flocculation.

This study has shown that flocculation has significant potential for the production of nutritious algal concentrates. In a study feeding juvenile bivalve molluscs diets of live and dried algae, Laing and Millican (1992) found oysters required a higher live algal diet component than the Manilla clam (*Tapes philippinarum*) to support growth comparable to a mixed live algal diet. This may mean that flocculated algal concentrates that have been demonstrated to be nutritious diets for juvenile Pacific oysters, could be even more nutritious to other aquaculture animals.

Published results on algal concentrates produced by centrifugation and their properties on storage show findings that are applicable to flocculated concentrates and could be used to improve their storage ability. In this study algal concentrates were stored in sealed, full containers in the dark at 4°C. Montaini *et al.* (1995), report that concentrates stored in this way lose their viability much more rapidly than those kept in cotton-plugged vials. Tredici *et al.* (1996) found that reducing the cell density of algal concentrates to 4 $g \cdot L^{-1}$ resulted in a concentrate of *Tetraselmis suecica* with no significant reduction in cell viability for 50 d of storage at 4°C. Malik (1995) added adsorption materials such as activated charcoal and trehalose to long-term standing cultures to increase cell survival and stability.

The use of algal concentrates could enable the more intensive rearing of aquaculture species by avoiding high rates of dilution caused by addition of large volumes of dilute live algal cultures. This has already been demonstrated for the intensive culture of the marine rotifer (*Brachionus rotundiformis*) with a 100-fold increase in production efficiency and a lower cost of production (Yoshimura *et al.*, 1996). Their use could also lower the nutrient load and bacterial numbers in larval culture by avoiding the addition

of algal culture media. Algal concentrates also make possible the feeding of non-traditional algal feed species to aquaculture animals. Abalone could now be fed non-benthic algal species by using partially deflocculated algal concentrates, which rapidly settle or could be incorporated in a binder such as agar.

7.5 Commercial Application of Algal Concentrates Produced by pH-Induced Flocculation

The production of algal concentrates using pH-induced flocculation has been costed according to the following parameters.

Flocculation parameters:

NaOH addition for flocculation at a final concentration of 8 mM

Polyelectrolyte (LT-25) added at 1 mL (0.05%)·L^{-1}

HCl addition to adjust pH, maximum of 500 mL to adjust pH of floc obtained from 1000 L of culture.

Chemical costs: Industrial grade NaOH and HCl

NaOH (Ajax Chemicals: 50% w/w, 200 L drum)	AU$ 2.00 per L
Polyelectrolyte LT-25 (Allied Colloids)	AU$ 7.50 per Kg
HCl 32% w/w (Ajax Chemicals: 240 Kg drum)	AU$ 0.76 per Kg

Chemical cost of flocculation and deflocculation of 1000 L of algal culture:

NaOH	AU$ 0.84	
Polyelectrolyte LT-25	AU$ 0.004	
HCl	AU$ 0.44	
Total cost =	AU$ 1.28	per 1000 L of culture

The polyelectrolyte is an insignificant cost with the overall cost split 65:35 between NaOH and HCl. Assuming a culture DW ~10 mg·100 mL^{-1}, then the cost per Kg DW is approximately AU$ 12.80. In 1990 the cost of hatchery algal production was up to US$ 400 per Kg (Coutteau and Sorgeloos, 1992). Assuming an exchange rate of US$ 1 = AU$ 1.6 and no increase in the production cost then flocculation could add 2% to this cost. However, a commercial producer of algal concentrates is likely to have production costs at the lower end of those reported by Coutteau and Sorgeloos (1992), within the range of US$ 50-100. Flocculation would then add between 8-16% to the cost of production. There is scope to reduce chemical costs by fine tuning the

flocculation process to match each algal species and culture conditions used by the producer.

Other costs associated with flocculation are labour, equipment, electricity, refrigeration and product shelf life. It is beyond the scope of this study to fully model all production costs but the following comments may be used as an indication of the relative significance of each cost area.

Labour: The flocculation method is relatively simple and could be undertaken by a competent, current employee of an aquaculture operation. Chemical handling is reduced by the use of solutions of chemicals and the addition of flocculant chemicals could be automated to reduce man-hours. The volume of each flocculation undertaken by a producer will influence the labour cost. The labour cost being largely independent of flocculation volume but dependent on the number of cycles.

Equipment: A pH meter is essential when re-adjusting the pH during de-flocculation. A chemical dosing pump is desirable but the process could be designed to operate without one. A venturi system could be used to dose the NaOH and polyelectrolyte. This would have the advantage of diluting the concentrated chemicals on-route to the flocculation vessel. A conical bottom flocculation tank would simplify the collection of the floc, but an existing culture vessel could be adapted.

Electricity: Electricity consumption for the flocculation process would be an insignificant addition to the normal consumption of a production facility. The main addition to electricity costs would result from the need to refrigerate the algal concentrates. This cost would be dependent on the production rate and holding period.

Refrigeration: All algal concentrates would require refrigerating to extend their shelf life. The size of the refrigeration unit will depend on the rate of production and on the holding period. As a guide, the maximum refrigerated volume will be 100 L per 10 000 L of algal production capacity.

Shelf life: All algal concentrates have a limited shelf life that will be dependent on the algal species. Minimising the cost associated with the loss of product quality is mainly a function of good housekeeping of the end user, which may be the manufacturer or a retail consumer. If the end user is the manufacturer then the costs associated with loss of product quality may be offset by the gains in productivity that result from the more efficient use of algal production capacity. This can occur by the use of algal concentrates in peak demand periods that were produced during off-peak periods. If the end user is a retail consumer then the producers costs associated with production are recovered in the premium price paid by the consumer for the convenience of an off-the-shelf product. For the purchaser the cost associated with a limited shelf life is minimised by accurately matching requirements to purchasing. The longer the shelf life the less impact it has on the viability of the manufacturing process. Methods to extend shelf life were not evaluated in this study and research in this area has the potential to further improve the economics of the flocculation process as a method to supply a nutritionally balanced off-the-shelf product for aquaculture consumers.

During completion of this PhD study a commercial prawn farm has incorporated this technology into their routine production of algae feed for prawn larvae. They have successfully fed algal concentrates to prawn larvae with no increase in mortality.

References

Adin, A. and Klein-Banay, C. (1986). Pretreatment of seawater by flocculation and settling for particulates removal. *Desalination* 58: 227-241.

Alaerts, G. and Van-Haute, A. (1981). Flocculation of brackish water from a tidal river. *Water Res.* 15: 517-523.

Albentosa, M, Pérez-Camacho, A., Labarta, U. and Fernández-Reiriz, M.J. (1996). Evaluation of live microalgal diets for the seed culture of *Ruditapes decussatus* using physiological and biochemical parameters. *Aquaculture* 148: 11-23.

Barillé, L., Prou, J., Héral, M. and Bourgrier, S. (1993). No influence of food quality, but ration-dependent retention efficiencies in the Japanese oyster *Crassostrea gigas*. *J. Exp. Mar. Biol. Ecol.* 171: 91-106.

Beim, A.A. and Beim, A.M. (1994). Comparative ecological-toxicological data on determination of maximum permissible concentrations (MPC) for several flocculants. *Environ. Technol.* 15: 195-198.

Ben-Amotz, A. and Avron, M. (1989). The biotechnology of mass culturing *Dunaliella* for products of commercial interest. In *Algal and cyanobacterial biotechnology*. Eds: R.C. Cresswell, T.A.V. Rees and N. Shah, Longman Scientific and Technical Press, pp. 90-114.

Ben-Amotz, A., Tornabene, T.G. and Thomas, W.H. (1985). Chemical profile of selected species of microalgae with emphasis on lipids. *J. Phycol.* 21: 72-81.

Benemann, J.R. (1992). Microalgae aquaculture feeds. *J. Appl. Phycol.* 4: 233-245.

Biedenbach, J.M., Smith, L.L. and Lawrence, A.L. (1990). Use of a new spray-dried algal product in Penaeid larviculture. *Aquaculture* 86: 249-257.

Bilanovic, D., Shelef, G. and Sukenik, A. (1988). Flocculation of microalgae with cationic polymers- Effects of medium salinity. *Biomass* 17: 65-76.

Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 912-917.

Booth, B.C., Lewin, J. and Lorenzen, C.J. (1988). Spring and summer growth rates of subarctic Pacific phytoplankton assemblages determined from carbon uptake and cell volume estimated using epifluorescence microscopy. *Mar. Biol.* 98: 287-298.

Brock, V. (1989). *Crassostrea gigas* (Thunberg) hepatopancreas-cellulase kinetics and cellulolysis of living monocellular algae with cellulose walls. *J. Exp. Mar. Biol. Ecol.* 128: 157-164.

Brown, M.R. (1991). The amino-acid and sugar composition of 16 species of microalgae used in mariculture. *J. Exp. Mar. Biol. Ecol.* 145: 79-99.

Brown, M.R. (1995). Effects of storage and processing on the ascorbic acid content of concentrates prepared from *Chaetoceros calcitrans*. *J. Appl. Phycol.* 7: 495-500.

Brown, M.R. and Farmer, C.L. (1994). Riboflavin content of six species of microalgae used in mariculture. *J. Appl. Phycol.* 6: 61-65.

Brown, M.R. and Jeffrey, S.W. (1992). Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1.Amino acids, sugars and pigments. *J. Exp. Mar. Biol. Ecol.* 161: 91-113.

Brown, M.R. and McCausland, M.A. (1988). *Increased production of juvenile Pacific Oysters (Crassostrea gigas) through supplementary feeding*. Final Report, Project no. 94/83 -Fisheries Research Development Council, 118 pp.

Brown, M.R. and Miller, K.A. (1992). The ascorbic acid content of eleven species of microalgae used in mariculture. *J. Appl. Phycol.* 4: 205-215.

Brown, M.R., Dunstan, G.A., Jeffrey, S.W., Volkman, J.K., Barret, S.M. and LeRoi, J.M. (1993). The influence of irradiance on the biochemical composition of the Prymnesiophyte *Isochrysis* sp. (clone T-iso). *J. Phycol.* 29: 601-612.

Brown, M.R., Dunstan, G.A., Norwood, S.J. and Miller, K.A. (1996). Effects of harvest stage and light on the biochemical composition of the diatom *Thalassiosira pseudonana*. *J. Phycol.* 32: 64-73.

Brown, M.R., Jeffery, S.W. and Garland, C.D. (1989). *Nutritional aspects of microalgae used in mariculture: a literature review*. CSIRO Marine Laboratories Report 205, CSIRO Hobart, Australia, 44 pp.

Brown, M.R., Jeffrey, S.W., Volkman, J.K. and Dunstan, G.A. (1997). Nutritional properties of microalgae for mariculture. *Aquaculture* 151: 315-331.

Buelna, G., Bhattarai, K.K., de-la-Noue, J. and Taiganides, E.P. (1990). Evaluation of various flocculants for the recovery of algal biomass grown on pig-waste. *Biol. Wastes* 31: 211-222.

Chen, F. and Johns. M.R. (1991). Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic *Chlorella sorokiniana*. *J. Appl. Phycol.* 3: 203-209.

Chu, F.E., Dupuy, J.L. and Webb, K.L. (1982). Polysaccharide composition of five algal species used as food for larvae of the American oyster, *Crassostrea virginica*. *Aquaculture* 29: 241-252.

Clayton, J.R. jr., Dortch, Q., Thoreson, S.S. and Ahmed, S.I. (1988). Evaluation of methods for the separation and analysis of proteins and free amino acids in phytoplankton samples. *J. Plankton Res.* 10: 341-358.

Cohen, Z. (1986). Products from microalgae. *In Handbook of Microalgal Mass Culture.* Ed. A. Richmond, CRC Press, Florida, pp. 421-454.

Coutteau, P. and Sorgeloos, P. (1992). The use of algal substitutes and the requirement for live algae in the hatchery and nursery rearing of bivalve molluscs: an international survey. *J. Shellf. Res.* 11: 467-476.

Coutteau, P., Lavens, P. and Sorgeloos, P. (1990). Baker's yeast as a potential substitute for live algae in aquaculture diets: Artemia as a case study. *J. World Aquaculture Soc.* 21: 1-9.

Crawford, R.M., Gardner, C. and Medlin, L.K. (1994). The genus *Attheya*. I. A description of four taxa, and the transfer of *Gonioceros septentrionalis* and *G. armatus*. *Diatom Research* 9: 27-51.

Cropp, C.M. and Garland, C.D. (1988). A scheme for the identification of marine bacteria. *Aust. Microbiologist* 9: 27-34.

Davis, B.D., Dulbecco, R., Eisen, H.N. and Ginsberg, H.S. (1980). The basis of chemotherapy. In: *Microbiology* (3rd Edition). Harper and Row, Maryland, pp. 111-126.

Donaldson, J. (1991). Commercial production of microalgae at Coast Oyster Company. In *Rotifer and microalgae culture systems.* Proc. US-Asia Workshop, Honolulu, HI, pp 229-236.

Dortch, Q., Clayton, J.R., Thoresen, S.S. and Ahmed, S.I. (1984). Species differences in accumulation of nitrogen pools in phytoplankton. *Mar. Biol.* 81: 237-250.

Dubois, M., Gillies, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for the determination of sugars and related substances. *Anal. Chem.* 28: 350-356.

Dunstan, G.A., Volkman, J.K., Barrett, S.M. and Garland, C.D. (1993). Changes in the lipid composition and maximisation of the polyunsaturated fatty acid content of three microalgae grown in mass culture. *J. Appl. Phycol.* 5: 71-83.

Dunstan, G.A., Volkman, J.K., Jeffrey, S.W. and Barrett, S.M. (1992). Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 2. Lipid classes and fatty acids. *J. Exp. Mar. Biol. Ecol.* 161: 115-134.

Enright, C.T., Newkirk, G.F., Craigie, J.S. and Castell, J.D. (1986a). Evaluation of phytoplankton as diets for juvenile *Ostrea edulis* L. *J. Exp. Mar. Biol. Ecol.* 96: 1-13.

Enright, C.T., Newkirk, G.F., Craigie, J.S. and Castell, J.D. (1986b). Growth of juvenile *Ostrea edulis* L. fed *Chaetoceros gracilis* Schütt of varied chemical composition. *J. Exp. Mar. Biol. Ecol.* 96: 15-26.

Epifanio, C.E. (1979). Growth in bivalve molluscs: nutritional effects of two or more species of algae in diets fed to the American oyster *Crassostrea virginica* (Gmelin) and the hard clam *Mercenaria mercenaria* (L.). *Aquaculture* 18: 1-12.

Estermann, R. (1994). Biological fuctions of carotenoids, *Aquaculture* 124: 219 (Abstract).

Ewing, R.D. and Finmore, F.J. (1970). Phospholipid metabolism during development of the brine shrimp *Artemia salina. Biochim. Biophys. Acta* 218: 474-481.

Fabregas, J., Herrero, C., Cabezas, B. and Abalde, J. (1986). Biomass production and biochemical composition in mass cultures of the marine microalgae *Isochrysis galbana* (Parke) at varying nutrient concentrations. *Aquaculture* 53: 101-113.

Flaak, A.R., and Epifanio, C.E. (1978). Dietary protein levels and growth of the oyster *Crassostrea virginica. Mar. Biol.* 45: 157-163.

Flynn, K.J. (1990). Composition of intracellular and extracellular pools of amino acids, and amino acid utilization of microalgae of different sizes. *J. Exp. Mar. Biol. Ecol.* 139: 151-166.

Flynn, K.J. and Al-Amoudi, O.A. (1988). Effects of N deprevation and darkness on composition of free amino acid pool in and on amino acid release from diatom *Phaeodactylum tricornutum* Bohlin. *J. Exp. Mar. Biol. Ecol.* 119: 131-143.

Gardner, C. and Crawford, R.M. (1992). A description of the diatom *Papiliocellulus simplex* sp. nov. (Cymatosiraceae, Bacillariophyta) using light and electron microscopy. *Phycologia* 31: 246-252.

Guillard, R.R.L., and Ryther, J.H. (1962). Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8: 229-239.

Hallegraeff, G.M. (1984). Species of the genus *Thalassiosira* in Australian waters. *Botanica Marina* 27: 495-513.

Hallegraeff, G.M. and Burford, M.A. (1996). Some new or little known nanoplankton diatoms cultured from tropical and subtropical Australian shelf waters. *Nova Hedwigia, Beiheft* 112: 329-342.

Hargraves, P.E. and Guillard, R.R.L. (1974). Structural and physiological observations on some small marine diatoms. *Phycologia* 13: 163-172.

Harrison, C. (1975). The essential amino acids of *Mytilus californianus. Veliger* 18; 189-193.

Harrison, P.J., Thompson, P.A. and Calderwood, G.S. (1990). Effects of nutrient and light limitation on the biochemical composition of phytoplankton. *J. Appl. Phycol.* 2: 45-56.

Hasdai, A. and Ben-Ghedalia, D. (1981). Sewage-grown algae as a source of supplementary nitrogen for ruminants. *J. Agric. Sci.* 97: 533-537.

Hasle, G.R. (1973). Some marine plankton genera of the diatom family Thalassiosiraceae. *Beiheft zur Nova Hedwigia* 45: 1-49.

Hasle, G.R. (1983). The marine planktonic diatoms *Thalassiosira oceanica* sp. nov. and *T. partheneia. J. Phycol.* 19: 220-229.

Hasle, G.R. and Syvertsen, E.E. (1997). Marine diatoms. In Identifying marine phytoplankton. Ed. C.R. Tomas, Academic Press, San Diego, USA, pp 5-385.

Hasle, G.R., Von Stosch, H.A. and Syvertsen, E.E. (1983). Cymatosiraceae, a new diatom family. *Bacillaria* 6: 9-156.

Hodgson, P.A., Henderson, R.J., Sargent, J.R. and Leftley, J.W. (1991). Patterns of variation in the lipid class and fatty acid composition of *Nannochloropsis oculata* (Eustigmatophyceae) during batch culture. *J. Appl. Phycol.* 3: 169-181.

Holland, D.L., and Hannant, P.J. (1974). Biochemical changes during growth of the spat of the oyster, *Ostrea edulis* L. *J. Mar. Biol. Ass. U.K.* 54: 1007-1016.

Holland, D.L., and Spencer, B.E. (1973). Biochemical changes in fed and starved oysters, *Ostrea edulis* L. during larval development, metamorphosis and early spat growth. *J. Mar. Biol. Ass. U.K.* 53: 287-298.

Jeffrey, S.W. and Humphrey, G.F. (1975). New spectrophotometric equations for determining chlorophylls *a, b,* c_1 and c_2 in higher plants, algae and natural phytoplankton. Biochem. *Physiol. Pflanzen* 167: 191-194.

Jeffrey, S.W., Mantoura, R.F.C. and Wright, S.W. (Eds.) (1997). *Phytoplankton pigments in oceanography: Guidelines to modern methods.* UNESCO Publishing, Paris, 661 pp.

James, C.M., Al-Hinty, S. and Salman, A.E. (1989). Growth and ω3 fatty acid and amino acid composition of microalgae under different temperature regimes. *Aquaculture* 77: 337-357.

Knauer, J. and Southgate, P.C. (1997). Assimilation of gelatin-acacia microencapsulated lipid by Pacific oyster (*Crassostrea gigas*) spat. *Aquaculture* 153: 291-300.

Knauer, J., Kerr, R.G., Lindley, D. and Southgate, P.C. (1998). Sterol metabolism of Pacific oyster (*Crassostrea gigas*) spat. *Comp. Biochem. Physiol.* 119B: 81-84.

Kochert, G. (1978). Quantitation of the macromolecular components of microalgae. In *Handbook of phycological methods: Physiological and biochemical methods.* Eds. J.A. Hellebust and J.S. Craigie, Cambridge University Press, Cambridge, pp 189-195.

Koopman, B. and Lincoln, E.P. (1983). Autoflotation harvesting of algae from high-rate pond effluents. *Agric. Wastes* 5: 231-246.

Kreeger, D.A. and Langdon, C.J. (1993). Effect of dietary protein content on growth of juvenile mussels, *Mytilus trossulus* (Gould 1850). *Biol. Bull.* 185: 123-139.

Laing, I. (1987). The use of artificial diets in rearing bivalve spat. *Aquaculture* 65: 243-249.

Laing, I. and Gil Verdugo, C. (1991). Nutritional value of spray-dried *Tetraselmis suecica* for juvenile bivalves. *Aquaculture* 92: 207-218.

Laing, I. and Millican, P.F. (1991). Dried-algal diets and indoor nursery cultivation of Manila clam juveniles. *Aquaculture* 95: 75-87.

Laing, I. and Millican, P.F. (1992). Indoor nursery cultivation of juvenile bivalve molluscs using diets of dried algae. *Aquaculture* 102: 231-243.

Laing, I., Child, A.R. and Janke, A. (1990). Nutritional value of dried algae for larvae of manila clam (*Tapes philippinarum*). *J. Mar. Biol. Ass. U.K.* 70: 1-12.

Langdon, C.J. and Bolton, E.T. (1984). A microparticulate diet for suspension-feeding bivalve mollusc, *Crassostrea virginica* (Gmelin). *J. Exp. Mar. Biol. Ecol.* 82: 239-258.

Langdon, C.J., and Waldock, M.J. (1981). The effect of algal and artificial diets on the growth and fatty acid composition of Crassostrea gigas spat. *J. Mar. Biol. Ass. U.K.* 61: 431-448.

Lange-Bertalot, H. (1976). Eine revision zur taxonomie der *Nitzschia lanceolate* Grunow. *Nova Hedwigia* 28: 302-303.

Lange-Bertalot, H. and Simonsen, R. (1978). A taxonomic revision of *the Nitzschia lanceolate* Grunow. *Bacillaria* 1: 11-113.

Langton, R.W. Winter, J.E and Roels, O.A. (1977). The effect of ration size on the growth and growth efficiency of the bivalave mollusc *Tapes japonica. Aquaculture* 12: 283-292.

Lavoie, A., de-la-Nouee, J. (1983). Harvesting microalgae with chitosan. *J. World Maricult. Soc.* 14: 685-694.

Leive, L.L. and Davis, B.D. (1980). Cell envelope; spores. In: *Microbiology* (3rd Edition). Eds. B.D. Davis, R. Dulbecco, H.N. Eisen and H.S. Ginsberg, Harper and Row, Maryland, pp. 71-110.

Lewis, T.E., Garland, C.D. and McMeekin, T.A. (1986). *Manual of hygiene for shellfish hatcheries*. University of Tasmania, Hobart, Tasmania, 45 pp.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

Lubián, L.M. (1989). Concentrating cultured marine microalgae with chitosan. *Aquacultural Engineering* 8: 257-265.

Lubzens, E., Gibson, O., Zmora, O. and Sukenik, A. (1995). Potential advantages of frozen algae (*Nannochloropsis* sp.) for rotifer (*Brachionus plicatilis*) culture. *Aquaculture* 133: 295-309.

Ma, J.C.M. and Jeffrey, L.M. (1978). Description and comparison of a new cleaning method of diatom frustules for light and electron microscope studies. *J. Microsc.* 112: 235-238.

Malik, K.A. (1995). A convenient method to maintain unicellular green algae for long times as standing liquid cultures. *J. Microbiological Methods* 22: 221-227.

Mayasich, S.A. and Smucker, R.A. (1986). Glycosidases in the American oyster, *Crassostrea virginica* Gmelin, digestive tract. *J. Exp. Mar. Biol. Ecol.* 95: 95-98.

McCausland, M.A., Brown, M.R., Barrett, S.M., Diemar, J. and Heasman, M.P. (1998). Evaluation of live and pasted microalgae as supplementary food for juvenile Pacific oysters (*Crassostrea gigas*). *Aquaculture* (submitted).

Millamena, O.M., Aujero, E.J. and Borlongan, I.G. (1990a). Techniques on algal harvesting and preservation for use in culture and as larval food. *Aquacultural Engineering* 9: 295-304.

Millamena, O.M., Peñaflorida, V.D. and Subosa, P.F. (1990b). The macronutrient composition of natural food organisms mass cultured as larval feed for fish and prawns. *Israeli J. Aquaculture-Bamidgeh* 42: 77-83.

Molina Grima, E., Sánchez Pérez, J.A., García Camacho, F., Acién Fernández, F.G., López Alonso, D., and Segura del Castillo, C.I. (1994). Preservation of the marine microalga, *Isochrysis galbana*: influence on the fatty acid profile. *Aquaculture* 123: 377-385.

Montaini, E., Chini Zittelli, G., Tredici, M.R., Molina Grima, E., Fernández Sevilla, J.M. and Sánchez Pérez, J.A. (1995). Long-term preservation of *Tetraselmis suecica*: influence of storage on viability and fatty acid profile. *Aquaculture* 134: 81-90.

Muller-Feuga, A., Robert, R., Moal, J., Marty, Y., Miner, P. and Mazuret, M. (1996). Institut Francais de Recherche pour l'Exploitation de la Mer (Brest). In *European Commission final report AIR1-CT92-0286(MANTA) -Microalgae*

Biomass from photobioreactors as food for fish and shellfish larvae (MANTA). European Commission, 138 pp.

Nell, J.A. and O'Connor, W.A. (1991). The evaluation of fresh algae and stored algal concentrates as a food source for Sydney rock oysters, *Saccostrea commercialis* (Iredale & Roughley), larvae. *Aquaculture* 99: 277-284.

Nell, J.A., Diemar, J.A. and Heasman, M.P. (1996). Food value of live yeasts and dry yeast-based diets fed to Sydney rock oyster *Saccostrea commercialis* spat. *Aquaculture* 145: 235-243.

O'Connor, W.A., Nell, J.A. and Diemar, J.A. (1992). The evaluation of twelve algal species as food for juvenile Sydney rock oysters *Saccostrea commercialis* (Iredale & Roughley). *Aquaculture* 108: 277-283.

Patterson, G.W. (1991). Sterols of algae. In *Physiology and biochemistry of sterols.* Eds. G.W. Patterson and W.D.Nes, American Oil Chemists Society, Champaign, Illinois, pp. 118-157.

Patterson, G.W., Tsitsa-Tzardis, E., Wikfors, G.H., Ghosh, P., Smith, B.C. and Gladu, P.K. (1994). Sterols of Eustigmatophtes. *Lipids* 29: 661-664.

Petrusevki, B., Van-Breemen, A.N. and Alaerts, G.J. (1995). Optimisation of coagulation conditions for direct filtration of impounded surface water. *Aqua* 44: 93-102.

Pillay, T.V.R. (1990). Nutrition and feeds. In *Aquaculture principles and practices.* Ed. T.V.R. Pillay, Fishing News Books, Cambridge, pp. 92-155.

Renaud, S.M., Parry, D.L., Thinh, L-V, Kuo, C., Padovan, A. and Sammy,N. (1991). Effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis* sp. and *Nannochloropsis oculata* for use in tropical aquaculture. *J. Appl. Phycol.* 3: 43-53.

Robert, R. and Trintignac, P. (1997). Substitutes for live microalgae in mariculture: a review. *Aquat. Living Resour.* 10: 315-327.

Rodhouse, P.G., Ottway, B. and Burnell, G.M. (1981). Bivalve production and food chain efficiency in an experimental nursery system. *J. Mar. Biol. Ass., U.K.* 61: 243-256.

Round, F.E., Crawford, R.M. and Mann, D.G. (1990). *The diatoms.* Cambridge University Press, Cambridge, 774 pp.

Sandbank, E. (1978). The utilization of microalgae as a feed for fish. *Ergeb. Limnol.* 11: 108-120.

Seguineau, C., Laschi-Loquerie, A., Moal, J. and Samain, J.F. (1996). Vitamin requirements in great scallop larvae. *Aquaculture International* 4: 315-324.

Shamsudin, L. (1992). Lipid and fatty acid composition of microalgae used in Malaysian aquaculture as live food for the early stage of penaeid larvae. *J. Appl. Phycol.* 4: 371-378.

Smith, R.R., Rumsey, G.L. and Scott, M.L. (1978). Heat increment associated with dietary protein, fat, carbohydrate and complete diets in salmonids: Comparative energetic efficiency. *J. Nutr.* 108: 1025-1032.

Speek, A.J., Schrijver, J. and Schreurs, W.H.P. (1984). Fluorometric determination of total vitamin C in whole blood by high-performance liquid chromatography with pre-column derivatization. *J. Chromatography* 305: 53-60.

Sripayatt, P (1982). Phytoplankton flocculation. *Thai-Fish. -Gaz.* 35: 609-611.

Sukenik, A., Bilanovic, D. and Shelef, G. (1988). Flocculation of microalgae in brackish and sea waters. *Biomass* 15: 187-199.

Thompson, P.A. and Harrison, P.J. (1992). Effects of monospecific algal diets of varying biochemical composition on the growth and survival of Pacific oyster *(Crassostrea gigas)* larvae. *Mar. Biol.* 113: 645-654.

Thompson, P.A., Guo, M. and Harrison, P.J. (1993). The influence of irradiance on the biochemical composition of three phytoplankton species and their nutritional value for larvae of the Pacific Oyster (*Crassostrea gigas*). *Mar. Biol.* 117: 259-268.

Throndsen, J. (1978). Preservation and Storage. In *Phytoplankton Manual.* Ed. A. Sournia, UNESCO, Paris, pp. 69-74.

Tredici, M., Montaini, E., Chini Zittelli, G. and Carobbi, S. (1996). Centro di Studio die Microorganismi Autotrofi (Florence). In *European Commission final report AIR1-CT92-0286(MANTA) -Microalgae Biomass from photobioreactors as food for fish and shellfish larvae (MANTA).* European Commission, 138 pp.

Urban, E.R. and Langdon, C.J. (1984). Reduction in costs of diets for the American oyster, *Crassostrea virginica* (Gmelin), by the use of non-algal supplements. *Aquaculture* 38: 277-291.

Utting, S.D. (1985). Influence of nitrogen availability on the biochemical composition of three unicellular marine algae of commercial importance. *Aquacult. Eng.* 4: 175-190.

Utting, S.D. (1986). A preliminary study on growth of *Crassostrea gigas* larvae and spat in relation to dietary protein. *Aquaculture* 56: 123-138.

Verity, P.G., Robertson, C.Y., Tronzo, C.R., Andrews, M.G., Nelson, J.R. and Sieracki, M.E. (1992). Relationship between cell volume and the carbon and nitrogen content of marine photosynthetic nanoplankton. *Limnol. Oceanogr.* 37: 1434-1446.

Viviers, J.M.P. and Briers, J.H. (1982). Harvesting of algae grown on raw sewage. *Water S.A.* 8: 178-186.

Vlaski, A., Van-Breemen, A.N. and Alaerts, G.J. (1996). The algal problem in the Netherlands from a water treatment perspective. *Aqua. J. Water Supply Technol.* 45: 184-194.

Vogel, A.I. (1978). Spectroanalytical methods. In *Vogel's Textbook of quantitative analytical analysis* 4th edition. Eds. J. Bassett, R.C. Denney, G.H. Jeffrey and J. Mendham, Longman, New York, pp. 691-772.

Volkman, J.K., Jeffrey, S.W., Nichols, P.D., Rogers, G.I. and Garland, C.D. (1989). Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *J. Exp. Mar. Biol. Ecol.* 128: 219-240.

Waldock, M.J. and Holland, D.L. (1984). Fatty acid metabolism in young oysters, Crassostrea gigas: polyunsaturated fatty acids. *Lipids* 19: 332-336.

Warren, C.E. and Davies, G.E. (1967). Laboratory studies on the feeding, bioenergetics, and growth of fish. *In The biological basis of freshwater fish production.* Ed. S.D. Gerking, Blackwell Scientific Publications, Oxford, England, pp. 175-214.

Watson, R.H., Jones, G.G. and Jones, B.L. (1986). Using concentrated algae for feeding oyster larvae. *J. Shellfish Res.*, 5: 136 (abstract).

Webb, K.L. and Chu, F.E. (1983). Phytoplankton as a food source for bivalve larvae. In *Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition, October 27-29, 1981 Lewes/Rehoboth Beach, Delware.* Eds G.D. Pruder, C.J.

Langdon and D.E. Conklin, Louisiana State University, Division of Continuing Education, pp. 272-291.

Whyte, J.C. (1987). Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. *Aquaculture* 60: 231-241.

Whyte, J.N.C., Bourne, N. and Hodgson, C.A. (1987). Assessment of biochemical composition and energy reserves in larvae of the scallop *Patinopecten yessoensis*. *J. Exp. Mar. Biol. Ecol.* 113: 113-124.

Whyte, J.N.C., Bourne, N. and Hodgson, C.A. (1989). Influence of algal diets on biochemical composition and energy reserves in *Patinopecten yessoensis* (Jay) larvae. *Aquaculture* 78: 333-347.

Whyte, J.N.C., Bourne, N. and Hodgson, C.A. (1990). Nutritional condition of rock scallop, *Crassadoma gigantea* (Gray), larvae fed mixed algal diets. *Aquaculture* 86: 25-40.

Wikfors, G.H., Gladu, P.K. and Patterson, G.W. (1991). In search of the ideal algal diet for oysters: Recent progress with emphasis on sterols. *J. Shellfish Res.* 10: 292 (abstract).

Wikfors, G.H., Patterson, G.W., Ghosh, P., Lewin, R.A., Smith, B.C. and Alix, J.H. (1996). Growth of post-set oysters *Crassostrea virginica*, on high-lipid strains of the algal flagellates *Tetraselmis* spp. *Aquaculture* 143: 411-419.

Withers, L.A. (1985). Cryropreservation and storage of germplasm. In *Plant cell culture (a practical approach)*. Ed. R.A. Dixon, IRL Press, Oxford, pp. 169-191.

Wright, S.W., Jeffrey, S.W., Mantoura, R.F.C., Llewellyn, C.A., Bjørnland, T., Repeta, D. and Welschmeyer, N. (1991). Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar. Ecol. Prog. Ser.* 77: 183-196.

Yoshimura, K., Hagiwara, A., Yoshimatsu, T. and Kitajima, C. (1996). Culture technology of marine rotifers and the implications for intensive culture of marine fish in Japan. *Mar. Freshwater Res.* 47: 217-222.

Appendix One

Growth Characteristics and Gross Composition of Australian Microalgae

Attheya septentrionalis (CS-425)

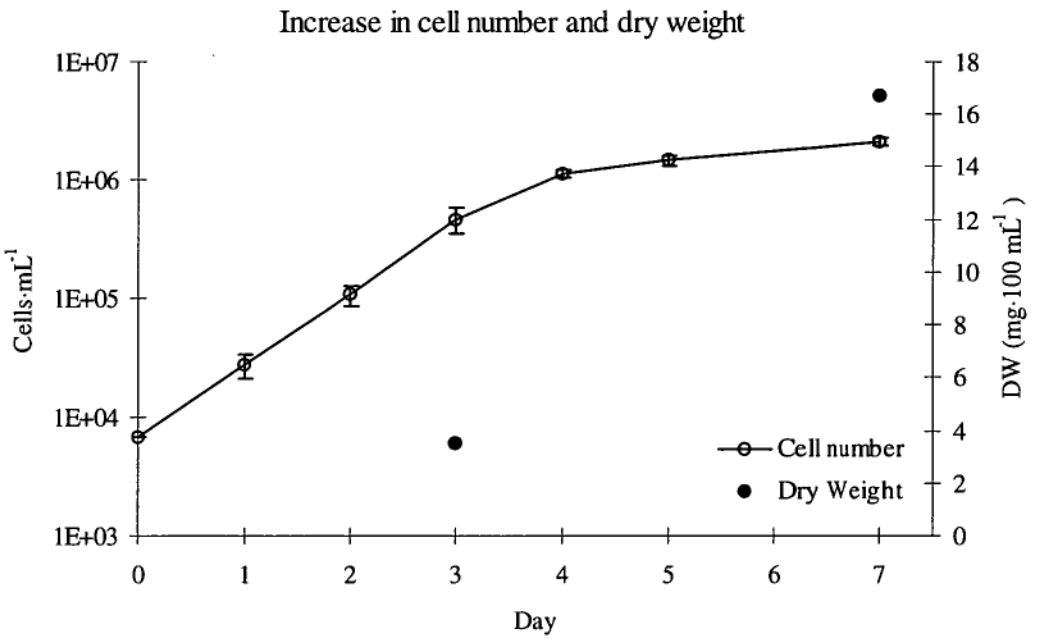
Growth data from triplicate cultures of *Attheya septentrionalis* (CS-425). Exponential growth samples were taken on day 3, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**1.41**
Growth rate	**2.04** divisions·d^{-1}
Generation time	**0.49** days
Carrying capacity	**2.08x10^{6}** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·cell^{-1})
0	6.67x10^{3} ± 0.00			
1	2.72x10^{4} ± 6.41x10^{3}			
2	1.07x10^{5} ± 2.06x10^{4}			
3	4.60x10^{5} ± 1.13x10^{5}	3.54 ± 0.50	2.63 ± 0.46	77
4	1.13x10^{6} ± 9.00x10^{4}			
5	1.47x10^{6} ± 1.59x10^{5}			
7	2.08x10^{6} ± 1.48x10^{5}	16.72 ± 1.77	15.25 ± 1.74	80



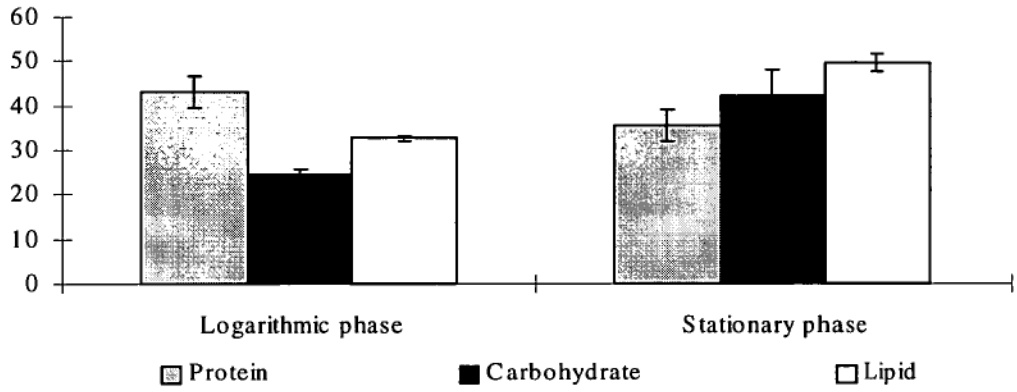
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	31.86 ± 3.72	18.05 ± 1.71	24.10 ± 0.49	26.13 ± 2.89	**100**
Stationary phase	32.43 ± 3.00	38.52 ± 5.78	45.30 ± 2.55	8.83 ± 1.55	**125**

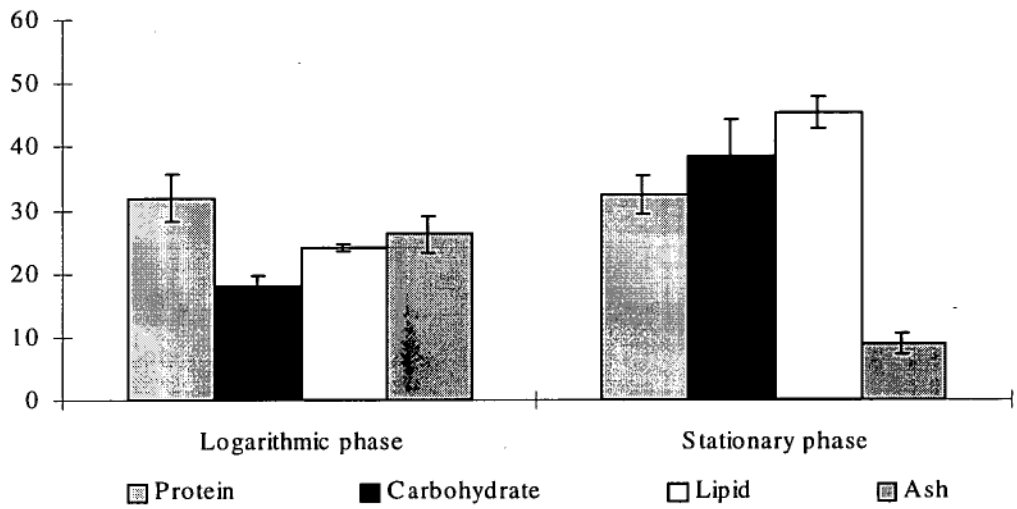
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	43.04 ± 3.40	24.40 ± 1.39	32.64 ± 0.62	**100**
Stationary phase	35.59 ± 3.56	42.23 ± 6.12	49.67 ± 2.10	**127**

Attheya septentrionalis (CS-425)

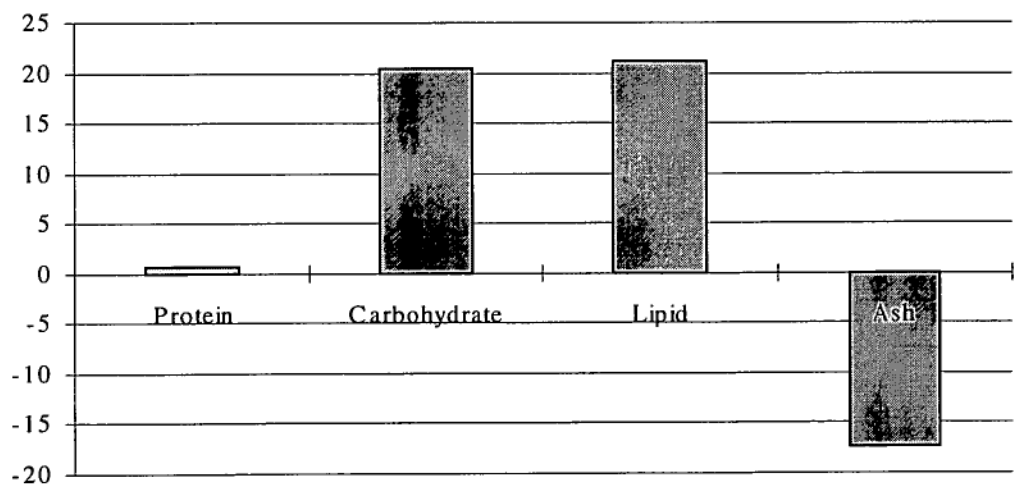
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Entomoneis cf. *punctulata* (CS-426)

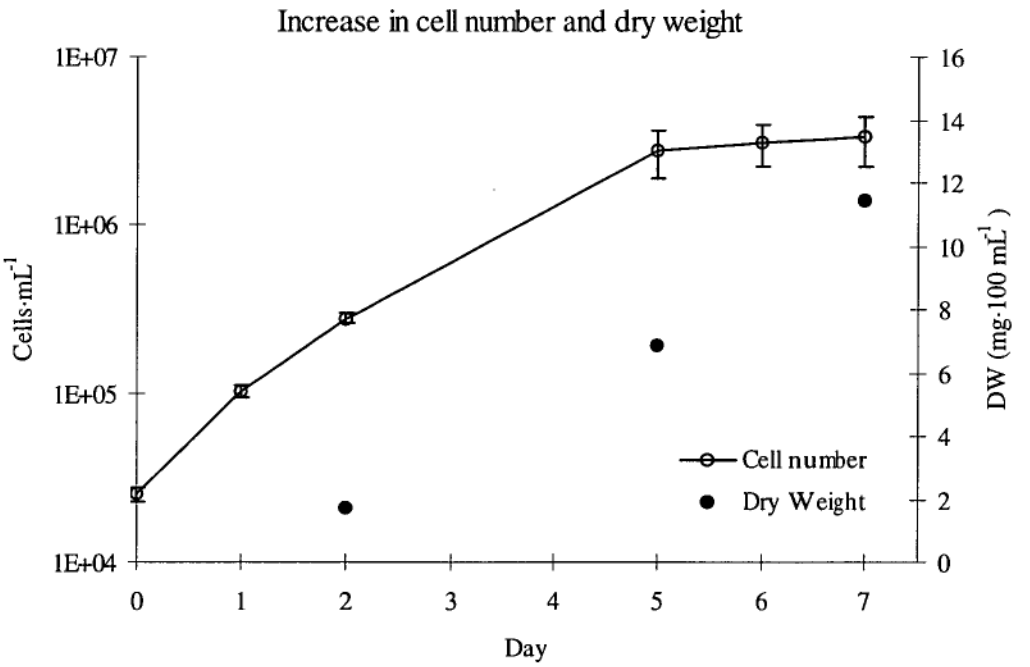
Growth data from triplicate cultures of *Entomoneis* cf. *punctulata* (CS-426). Exponential growth samples were taken on day 2, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**1.20**
Growth rate	**1.73** divisions·d^{-1}
Generation time	**0.58** days
Carrying capacity	**$3.05x10^6$** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·$cell^{-1}$)
0	$2.52x10^4 \pm 2.25x10^3$			
1	$1.04x10^5 \pm 8.94x10^3$			
2	$2.78x10^5 \pm 1.89x10^4$	1.71 ± 0.25	1.11 ± 0.12	61
5	$2.73x10^6 \pm 8.63x10^5$	6.84 ± 1.07	5.47 ± 0.71	25
6	$3.11x10^6 \pm 8.73x10^5$			
7	$3.32x10^6 \pm 1.13x10^6$	11.45 ± 3.01	9.78 ± 2.66	34



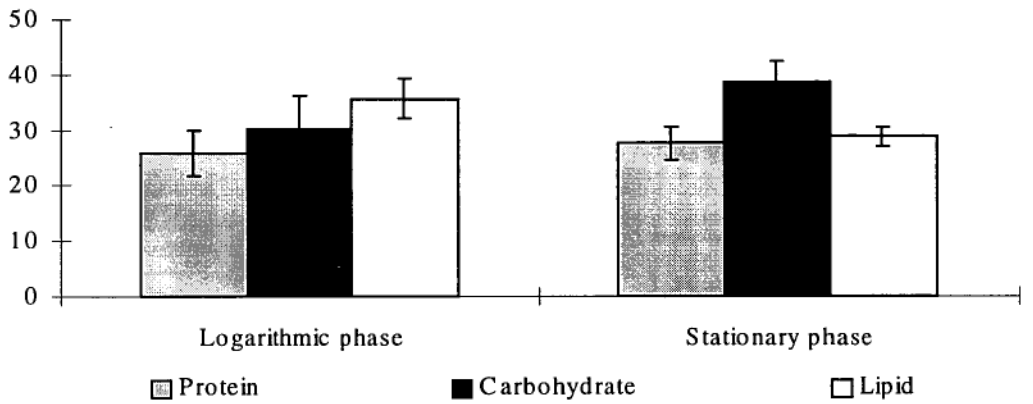
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	16.87 ± 3.31	19.72 ± 4.81	28.60 ± 3.72	34.94 ± 2.97	**100**
Stationary phase	23.50 ± 2.43	32.97 ± 3.06	24.63 ± 1.33	14.67 ± 1.31	**96**

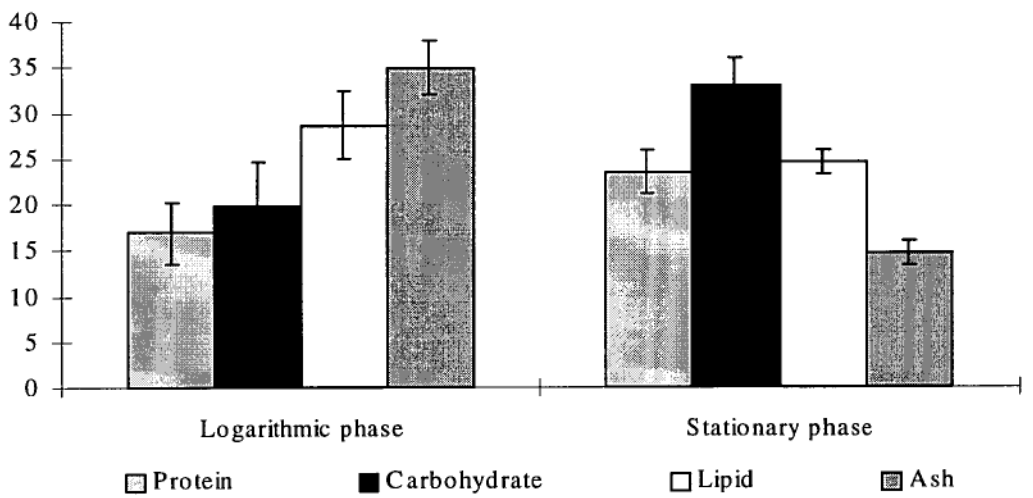
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	25.80 ± 3.99	30.12 ± 6.19	35.59 ± 3.61	**92**
Stationary phase	27.55 ± 3.06	38.65 ± 3.67	28.87 ± 1.79	**95**

Entomoneis cf. *punctulata* (CS-426)

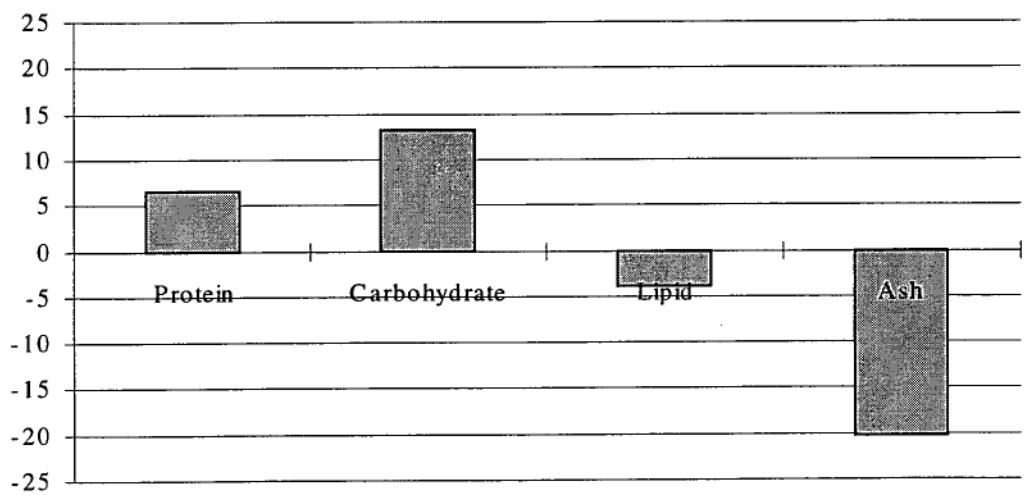
Composition (%AFDW) of logarithmic and stationary phase cultures



Composition (%DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Extubocellulus spinifera (CS-428)

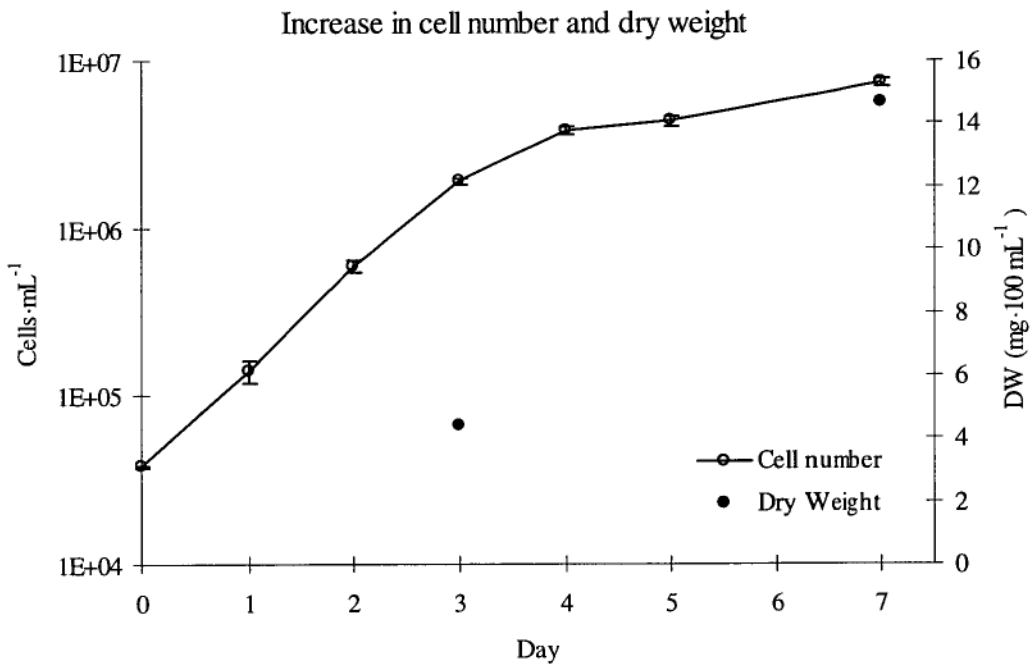
Growth data from triplicate cultures of *Extubocellulus spinifera* (CS-428). Exponential growth samples were taken on day 3, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**1.30**
Growth rate	**1.88** divisions·d^{-1}
Generation time	**0.53** days
Carrying capacity	**$7.33x10^6$** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·$cell^{-1}$)
0	$3.80x10^4 \pm 4.28x10^2$			
1	$1.40x10^5 \pm 2.14x10^4$			
2	$5.92x10^5 \pm 4.86x10^4$			
3	$1.91x10^6 \pm 8.08x10^4$	4.37 ± 0.62	2.88 ± 0.46	23
4	$3.81x10^6 \pm 2.04x10^5$			
5	$4.34x10^6 \pm 2.68x10^5$			
7	$7.33x10^6 \pm 4.06x10^5$	14.67 ± 2.51	12.64 ± 2.35	20



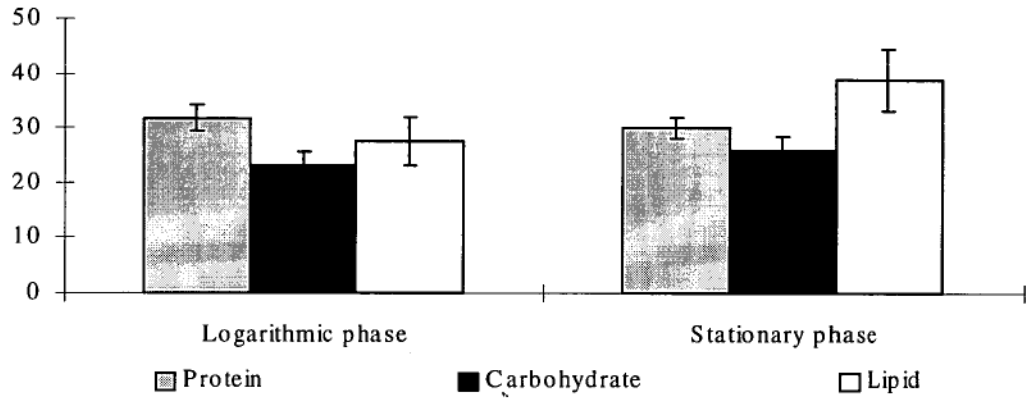
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	20.96 ± 1.73	15.27 ± 1.93	18.15 ± 3.33	34.07 ± 1.82	**88**
Stationary phase	25.87 ± 1.32	22.28 ± 2.69	33.39 ± 4.22	13.98 ± 1.99	**96**

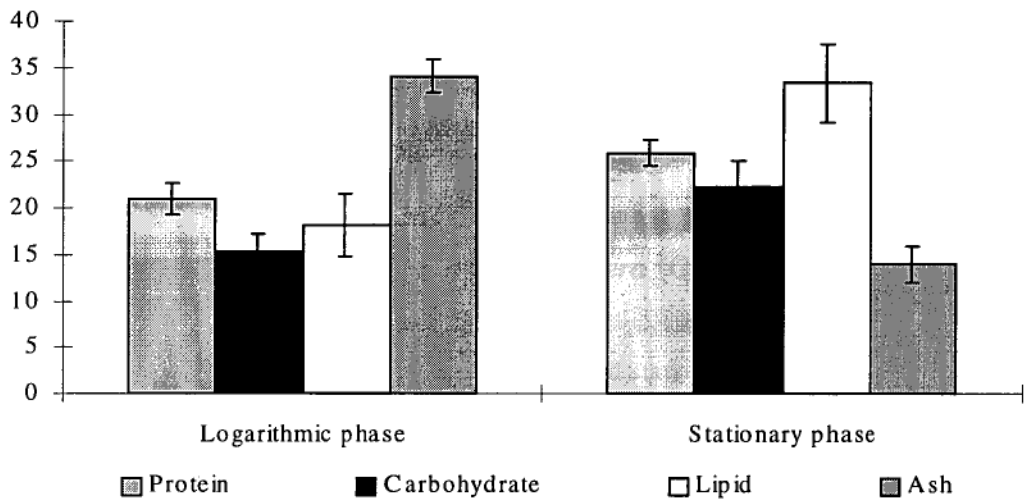
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	31.79 ± 2.35	23.13 ± 2.38	27.46 ± 4.35	**82**
Stationary phase	30.09 ± 1.80	25.88 ± 2.68	38.91 ± 5.68	**95**

Extubocellulus spinifera (CS-428)

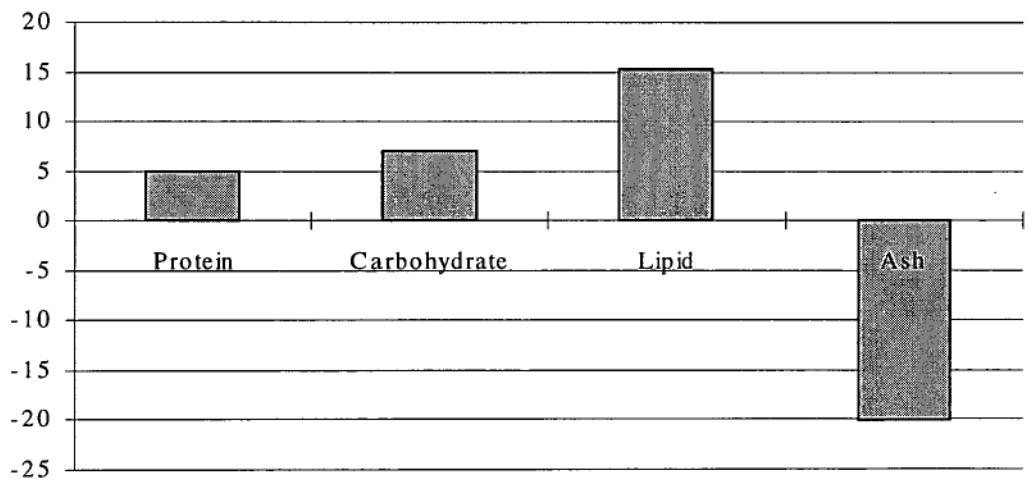
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Minidiscus trioculatus (CS-435)

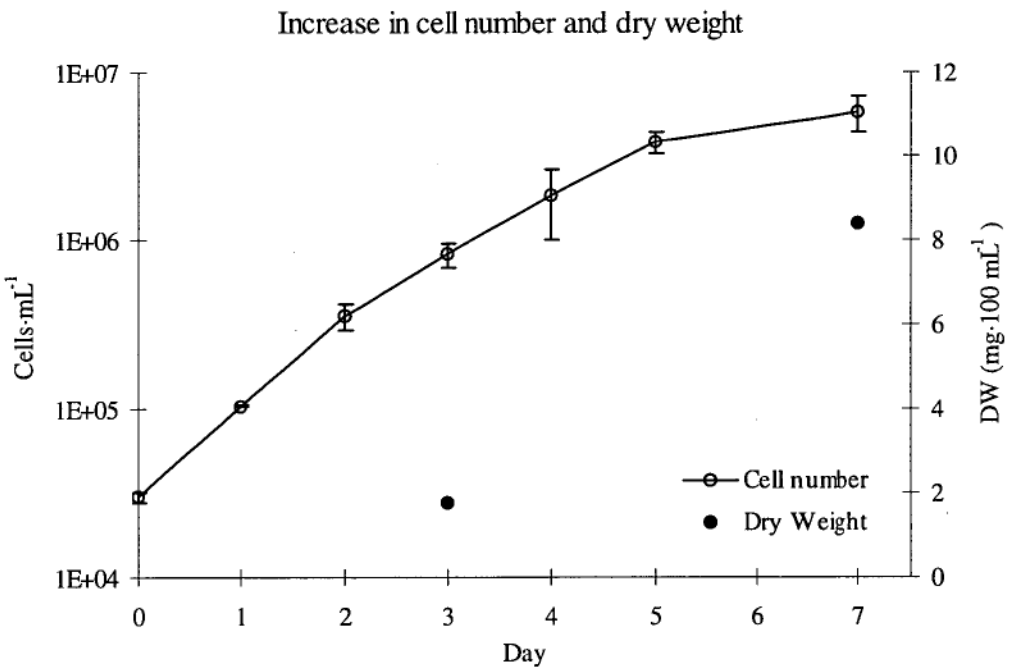
Growth data from triplicate cultures of *Minidiscus trioculatus* (CS-435). Exponential growth samples were taken on day 3, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**0.97**
Growth rate	**1.40** divisions·d^{-1}
Generation time	**0.71** days
Carrying capacity	**5.83x10^{6}** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·cell^{-1})
0	2.96x10^{4} ± 1.70x10^{3}			
1	1.03x10^{5} ± 1.16x10^{3}			
2	3.53x10^{5} ± 6.10x10^{4}			
3	8.23x10^{5} ± 1.36x10^{5}	1.78 ± 0.02	1.10 ± 0.07	22
4	1.82x10^{6} ± 8.14x10^{5}			
5	3.82x10^{6} ± 5.37x10^{5}			
7	5.83x10^{6} ± 1.45x10^{6}	8.37 ± 0.94	6.61 ± 0.70	14



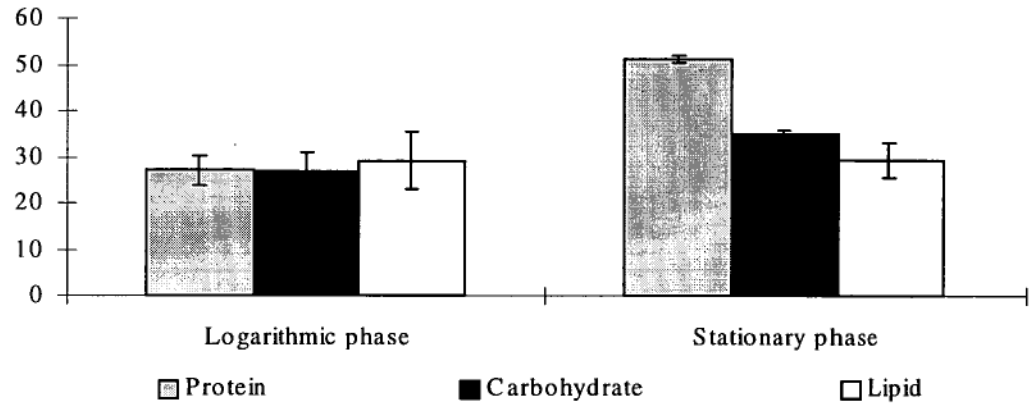
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	16.72 ± 3.04	16.56 ± 3.31	17.24 ± 3.55	38.28 ± 4.63	**89**
Stationary phase	40.59 ± 1.05	27.87 ± 0.48	23.28 ± 3.21	20.95 ± 1.91	**113**

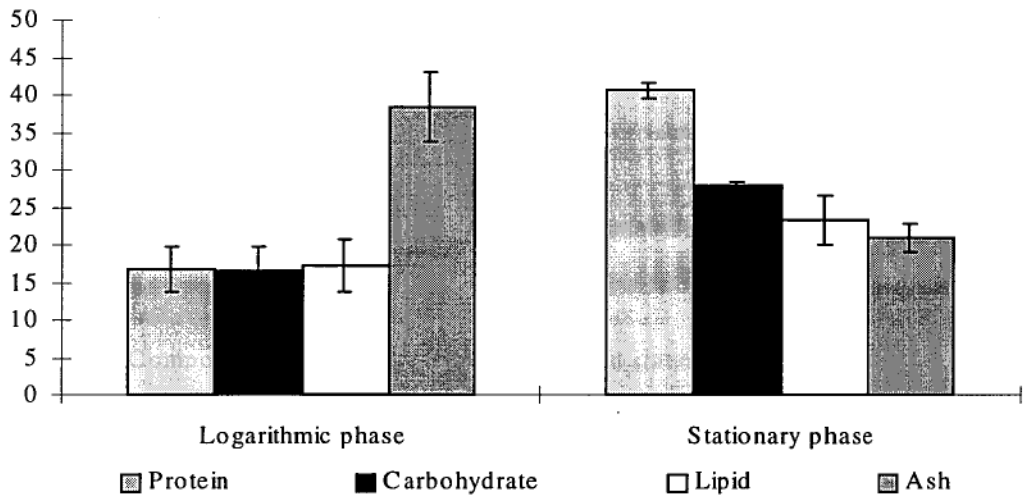
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	26.98 ± 3.19	26.73 ± 4.03	29.23 ± 6.29	**83**
Stationary phase	51.35 ± 0.81	35.26 ± 0.41	29.43 ± 3.86	**116**

Minidiscus trioculatus (CS-435)

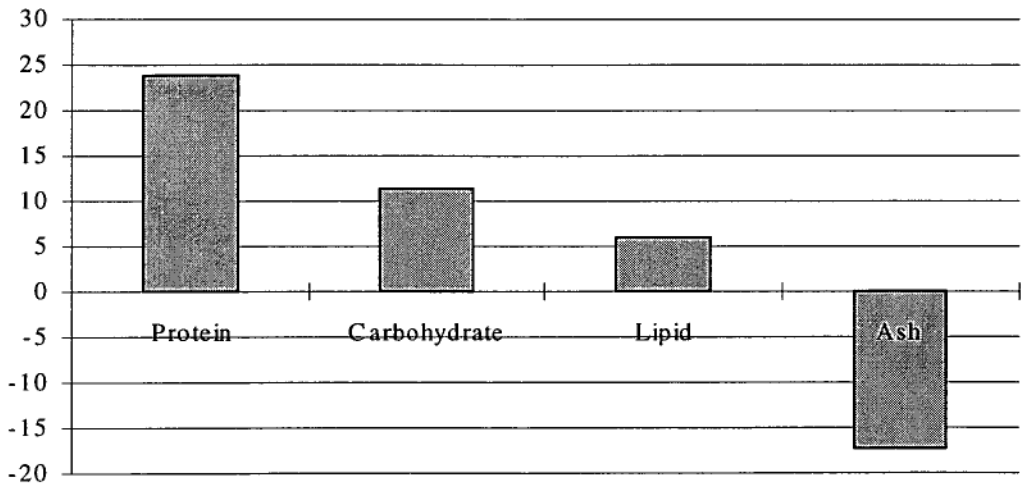
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Minidiscus trioculatus (CS-432)

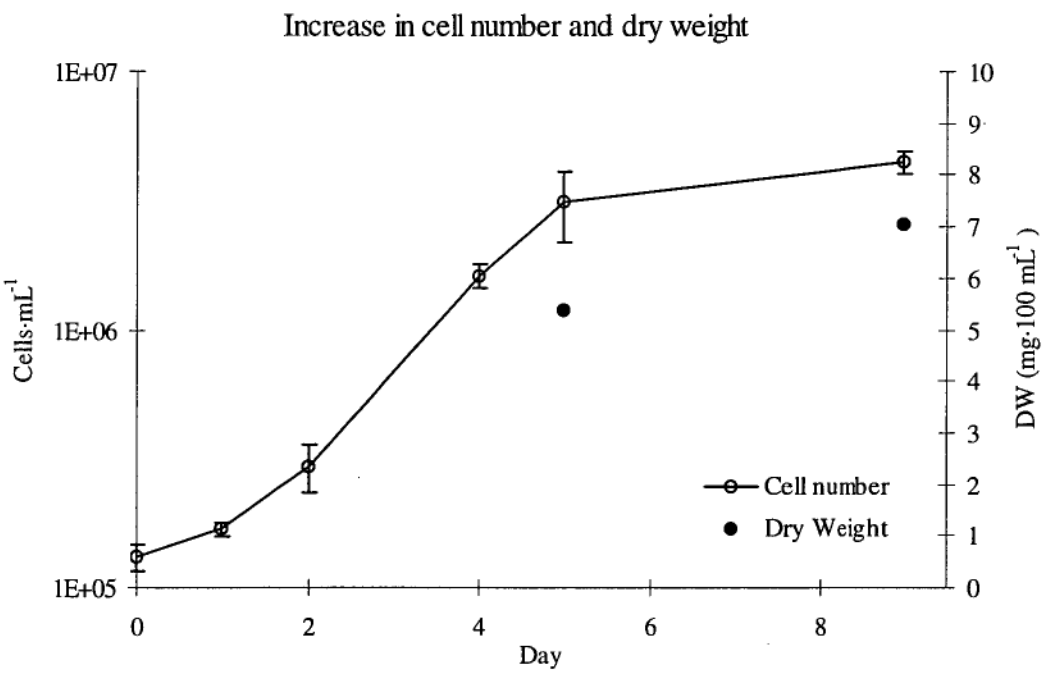
Growth data from triplicate cultures of *Minidiscus trioculatus* (CS-432). Exponential growth samples were taken on day 5, stationary phase samples on day 9.

Growth Statistics

Growth rate (μ)	**0.73**
Growth rate	**1.05** divisions·d^{-1}
Generation time	**0.95** days
Carrying capacity	**$3.78x10^6$** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·$cell^{-1}$)
0	$1.30x10^5 \pm 1.56x10^4$			
1	$1.68x10^5 \pm 1.06x10^4$			
2	$2.93x10^5 \pm 6.13x10^4$			
4	$1.61x10^6 \pm 1.68x10^5$			
5	$3.12x10^6 \pm 9.50x10^5$	5.37 ± 0.80	3.78 ± 0.60	17
9	$4.45x10^6 \pm 4.27x10^5$	7.05 ± 1.05	5.38 ± 1.02	16



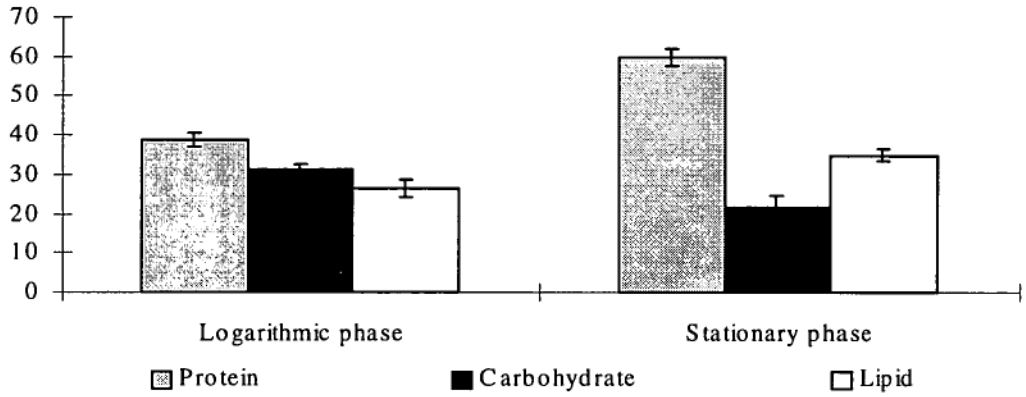
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	27.14 ± 0.90	21.98 ± 1.43	21.99 ± 1.53	29.68 ± 2.90	**101**
Stationary phase	45.42 ± 3.74	16.56 ± 3.04	31.34 ± 3.11	24.07 ± 3.54	**117**

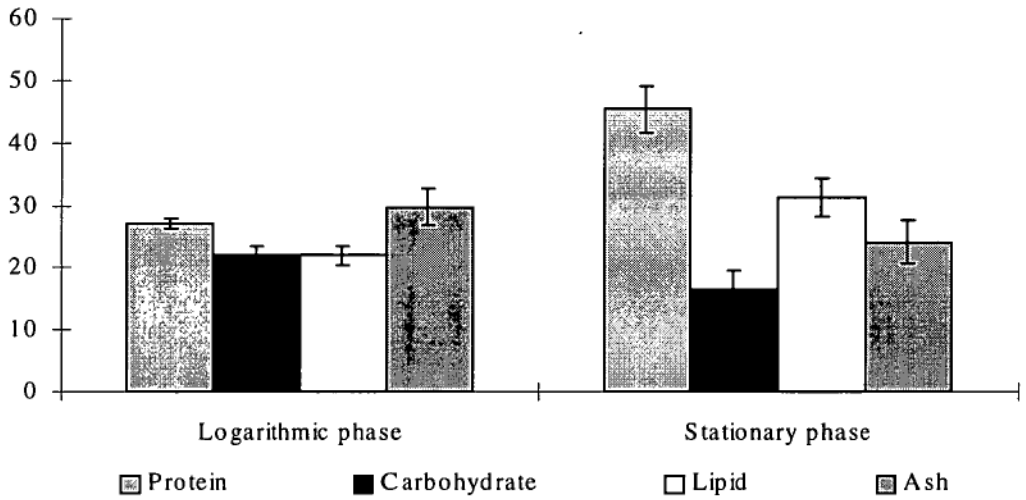
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	38.63 ± 1.83	31.25 ± 1.45	26.48 ± 2.33	**96**
Stationary phase	59.75 ± 2.21	21.71 ± 3.08	34.83 ± 1.56	**116**

Minidiscus trioculatus (CS-432)

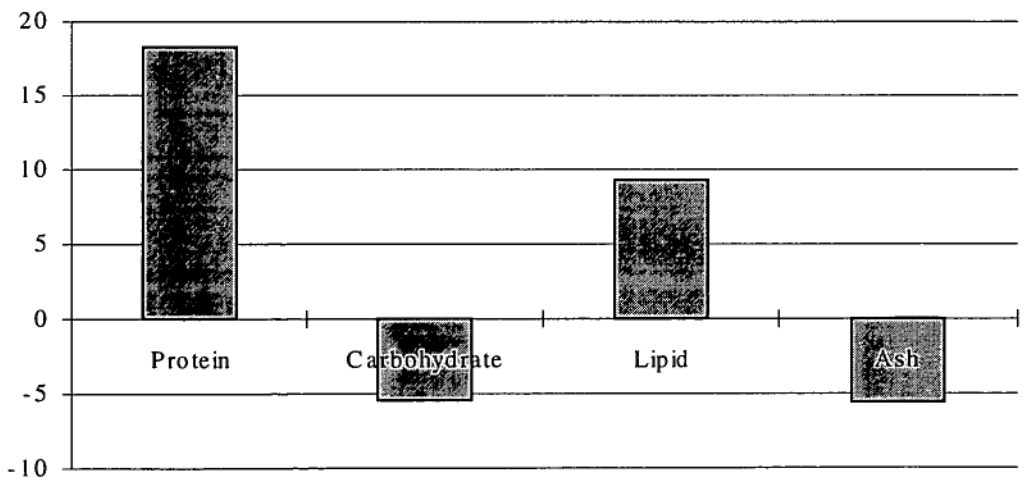
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Nitzschia cf. *paleacea* (CS-429)

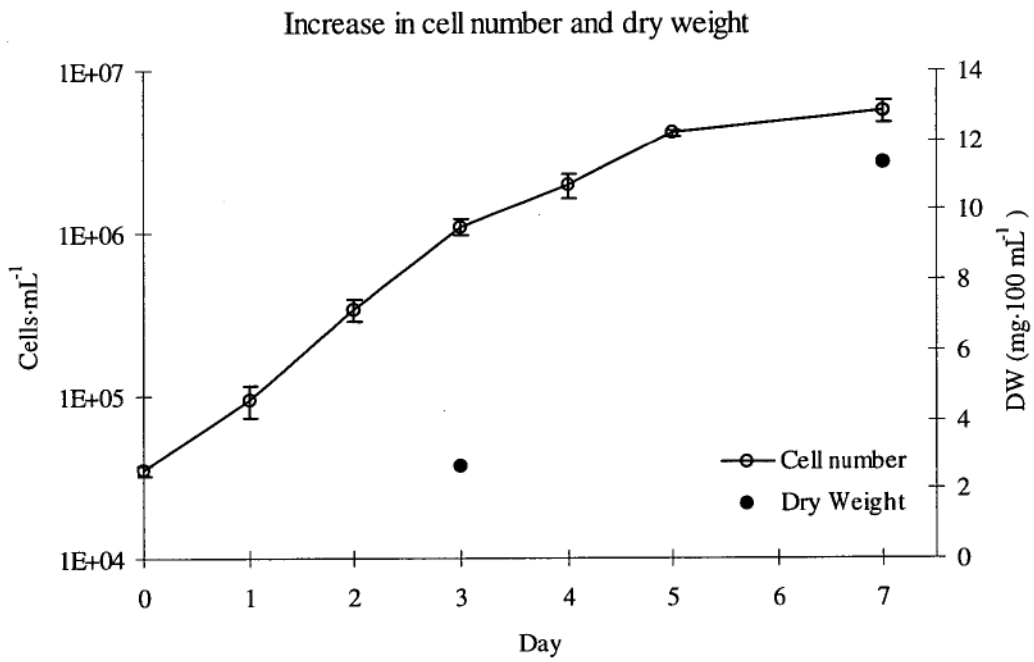
Growth data from triplicate cultures of *Nitzschia* cf. *paleacea* (CS-429). Exponential growth samples were taken on day 3, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**0.95**
Growth rate	**1.37** divisions·d^{-1}
Generation time	**0.73** days
Carrying capacity	**$4.87x10^6$** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·$cell^{-1}$)
0	$3.49x10^4 \pm 2.58x10^3$			
1	$9.44x10^4 \pm 2.08x10^4$			
2	$3.41x10^5 \pm 5.14x10^4$			
3	$1.10x10^6 \pm 1.18x10^5$	2.63 ± 0.11	1.86 ± 0.13	24
4	$1.96x10^6 \pm 3.50x10^5$			
5	$4.10x10^6 \pm 1.46x10^5$			
7	$5.63x10^6 \pm 8.28x10^5$	11.35 ± 0.61	9.36 ± 0.44	20



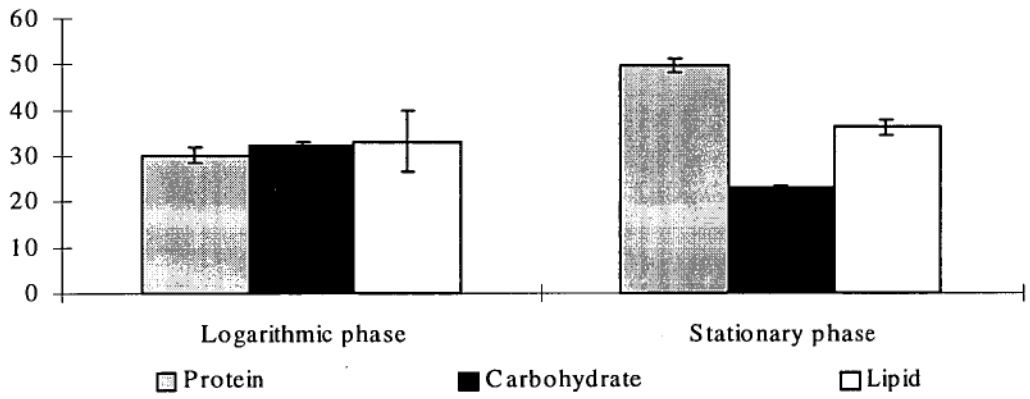
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	21.28 ± 1.18	22.88 ± 0.86	23.46 ± 4.35	29.21 ± 2.07	**97**
Stationary phase	40.98 ± 1.57	19.03 ± 0.21	29.93 ± 1.65	17.50 ± 1.03	**107**

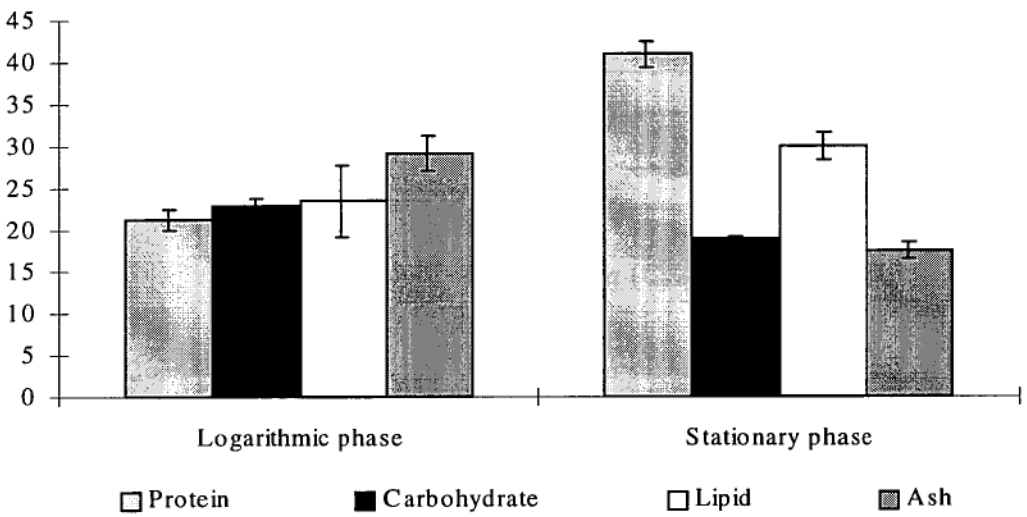
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	30.07 ± 1.70	32.33 ± 0.80	33.22 ± 6.70	**96**
Stationary phase	49.66 ± 1.54	23.07 ± 0.49	36.28 ± 1.79	**109**

Nitzschia cf. *paleacea* (CS-429)

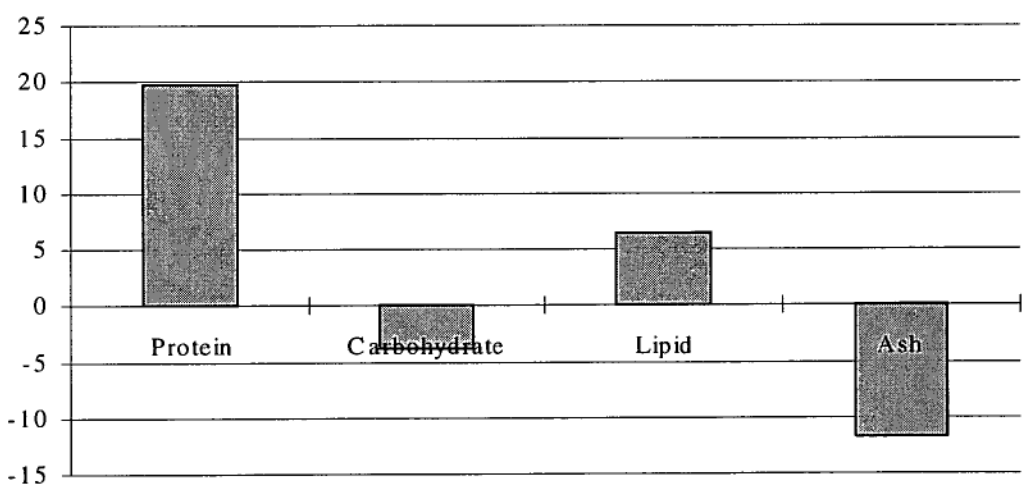
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Nitzschia cf. *paleacea* (CS-430)

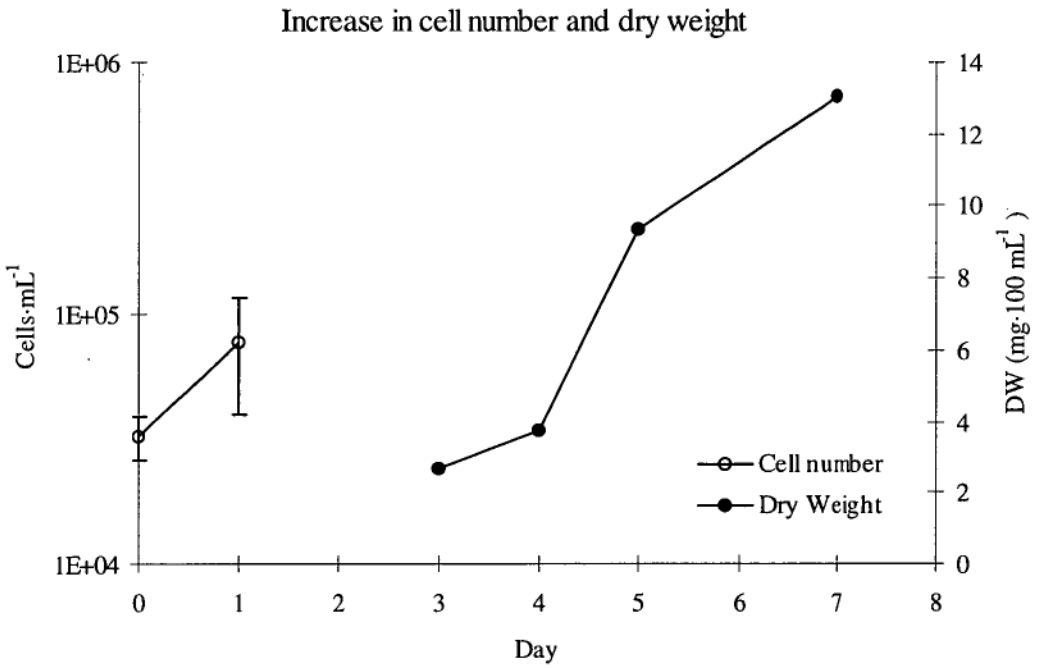
Growth data from triplicate cultures of *Nitzschia* cf. *paleacea* (CS-430). Exponential growth samples were taken on day 4, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**0.62**
Growth rate	**0.90** divisions·d^{-1}
Generation time	**1.11** days
Carrying capacity	**13.05** mg·100 mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW (mg·100 mL^{-1} ± S.D.)	Cell mass (pg·$cell^{-1}$)
0	$3.26x10^4 \pm 6.32x10^3$			
1	$7.78x10^4 \pm 3.77x10^4$			
2				
3		2.70 ± 0.56	1.92 ± 0.39	
4		3.77 ± 1.14	2.90 ± 0.81	
5		9.36 ± 2.86	7.56 ± 2.48	
7		13.05 ± 2.56	11.13 ± 2.21	



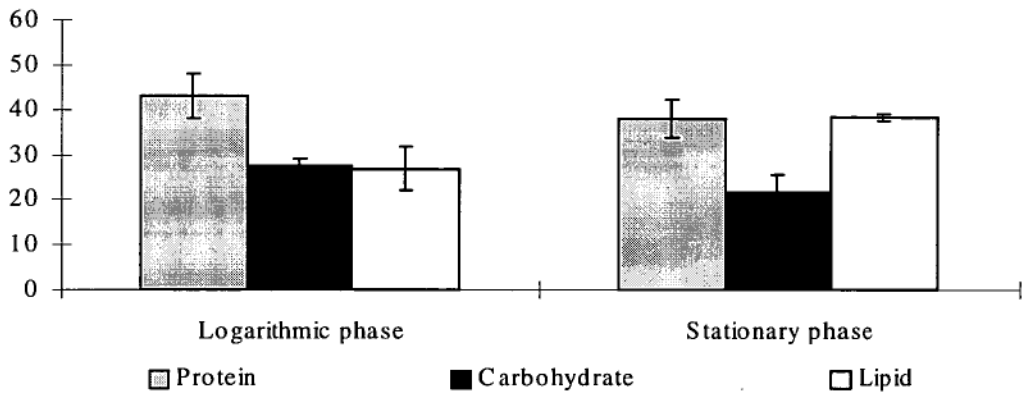
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	33.19 ± 4.63	21.11 ± 1.90	20.80 ± 4.28	22.85 ± 2.16	**98**
Stationary phase	32.26 ± 3.91	18.38 ± 3.55	32.61 ± 0.58	14.76 ± 1.31	**98**

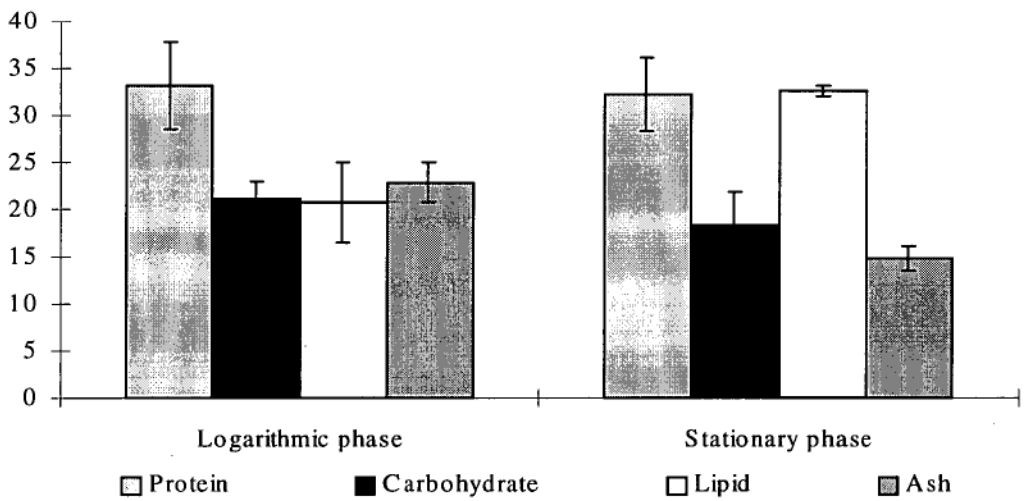
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	42.94 ± 4.90	27.34 ± 1.84	26.88 ± 4.86	**97**
Stationary phase	37.82 ± 4.27	21.55 ± 4.00	38.27 ± 0.84	**98**

Nitzschia cf. *paleacea* (CS-430)

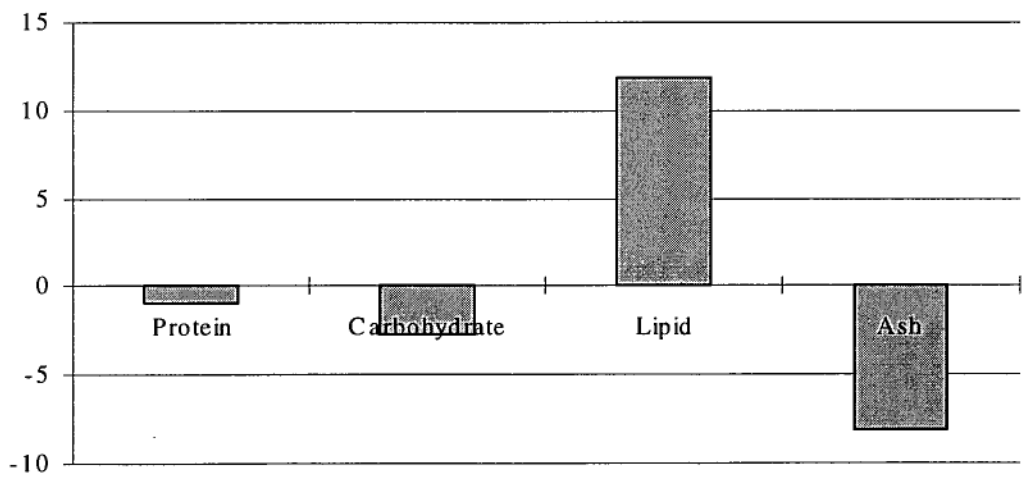
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Nitzschia cf. *paleacea* (CS-433)

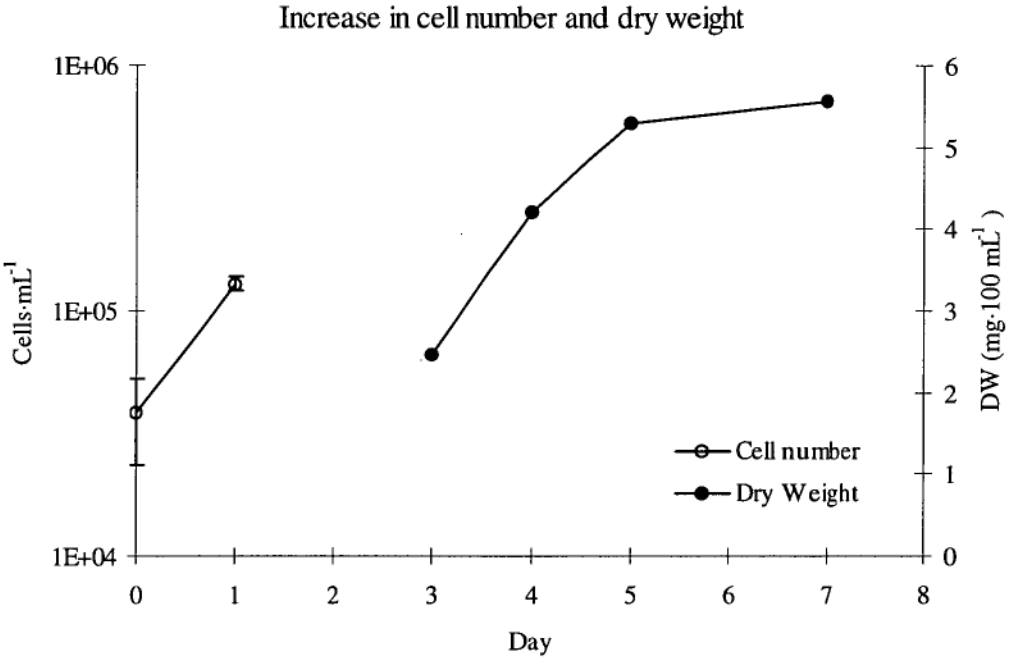
Growth data from triplicate cultures of *Nitzschia* cf. *paleacea* (CS-433). Exponential growth samples were taken on day 4, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**0.38**
Growth rate	**0.55** divisions·d^{-1}
Generation time	**1.81** days
Carrying capacity	**5.42** mg·100 mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW (mg·100 mL^{-1} ± S.D.)	Cell mass (pg·cell^{-1})
0	$3.80 \times 10^4 \pm 1.45 \times 10^4$			
1	$1.29 \times 10^5 \pm 8.01 \times 10^3$			
2				
3		2.46 ± 0.32	1.28 ± 0.13	
4		4.21 ± 0.26	2.39 ± 0.16	
5		5.28 ± 1.41	3.02 ± 0.71	
7		5.56 ± 1.27	3.40 ± 0.74	

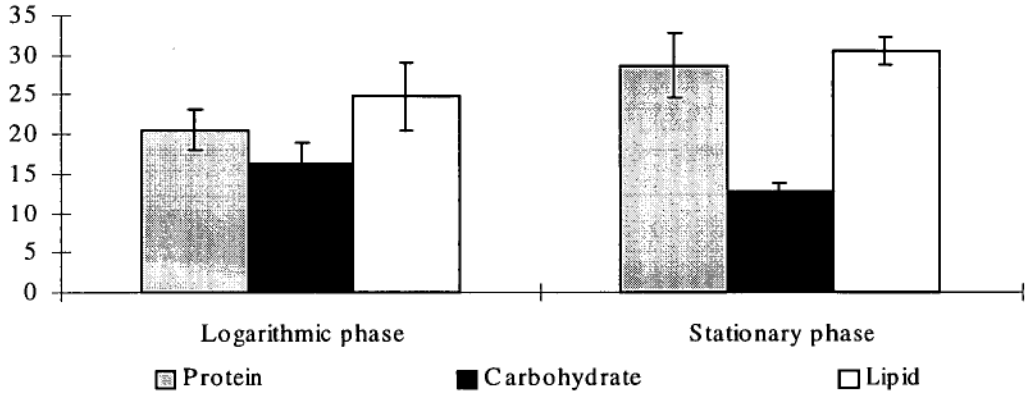


Percentage Composition

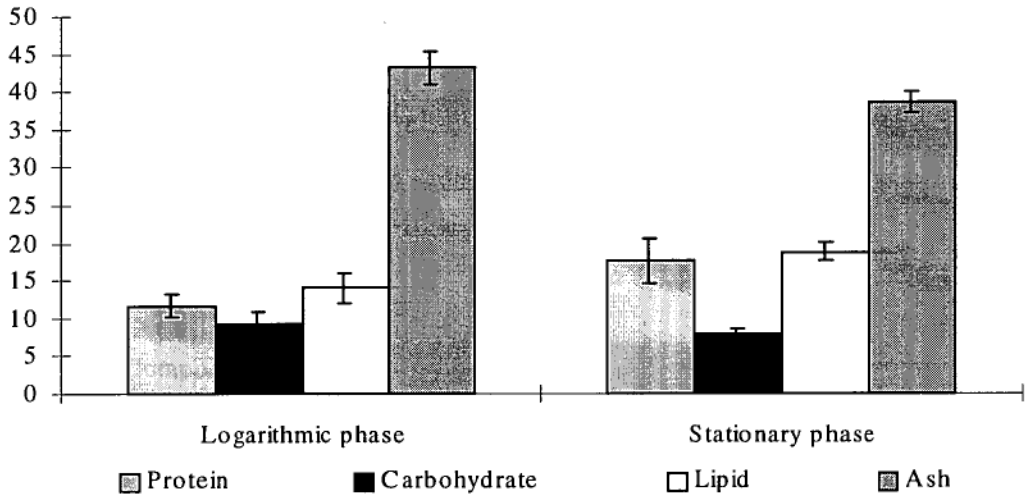
Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	11.68 ±1.61	9.26 ±1.58	14.02 ±2.04	43.26 ±2.21	**78**
Stationary phase	17.66 ±2.98	7.80 ±0.71	18.81 ±1.28	38.64 ±1.37	**83**

Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	20.56 ±2.50	16.29 ±2.54	24.82 ±4.35	**62**
Stationary phase	28.73 ±4.18	12.70 ±1.12	30.64 ±1.74	**72**

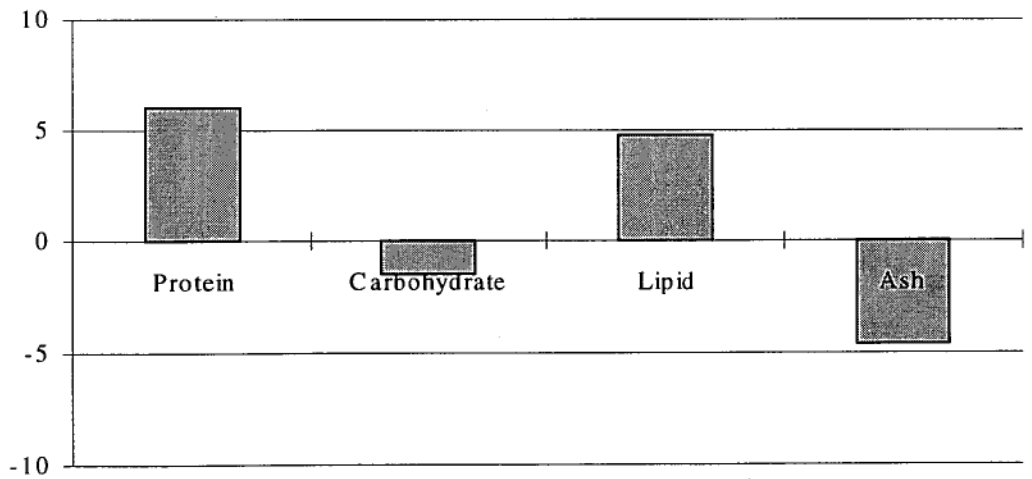
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Papiliocellulus simplex (CS-431)

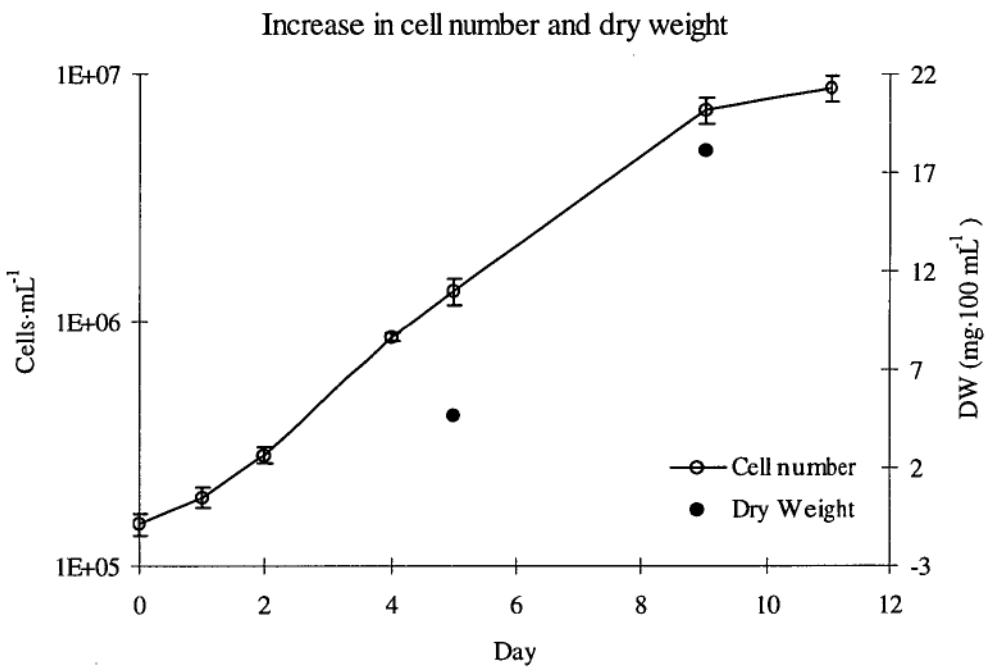
Growth data from triplicate cultures of *Papiliocellulus simplex* (CS-431). Exponential growth samples were taken on day 5, stationary phase samples on day 9.

Growth Statistics

Growth rate (μ)	**0.46**
Growth rate	**0.66** divisions·d^{-1}
Generation time	**1.51** days
Carrying capacity	**8.75x10^{6}** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·cell^{-1})
0	1.49x10^{5} ± 1.57x10^{4}			
1	1.90x10^{5} ± 1.76x10^{4}			
2	2.82x10^{5} ± 2.28x10^{4}			
4	8.47x10^{5} ± 3.21x10^{4}			
5	1.31x10^{6} ± 1.63x10^{5}	4.62 ± 0.34	4.19 ± 0.34	35
9	7.08x10^{6} ± 8.78x10^{5}	18.09 ± 2.38	16.26 ± 2.31	26
11	8.75x10^{6} ± 1.00x10^{6}			



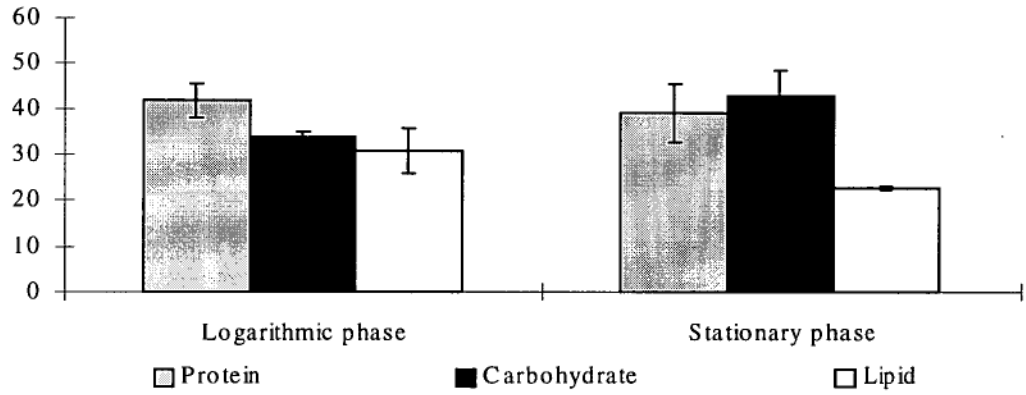
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	37.78 ± 3.29	30.73 ± 0.78	27.87 ± 4.37	9.33 ± 1.02	**106**
Stationary phase	35.10 ± 5.38	38.50 ± 5.75	20.56 ± 0.26	10.20 ± 0.95	**104**

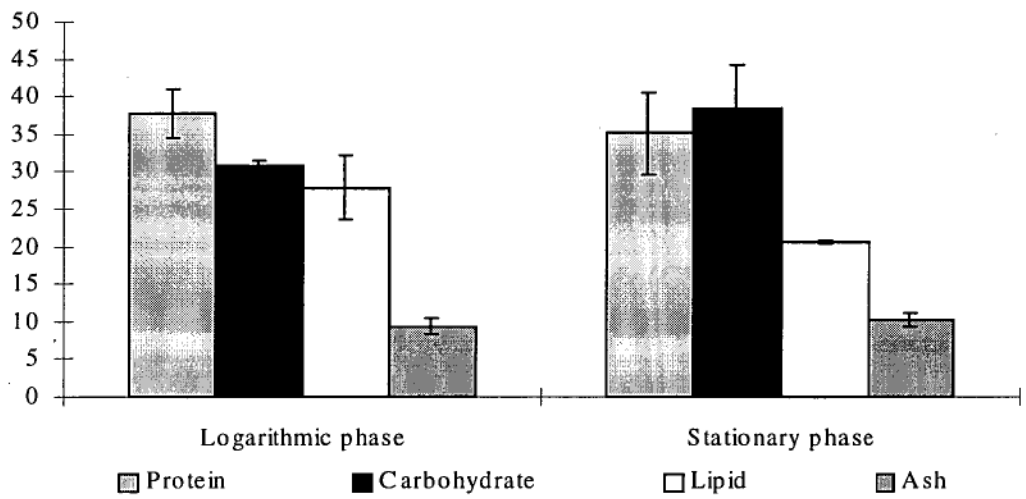
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	41.69 ± 3.82	33.90 ± 1.06	30.76 ± 5.05	**106**
Stationary phase	39.13 ± 6.41	42.83 ± 5.95	22.90 ± 0.39	**105**

Papiliocellulus simplex (CS-431)

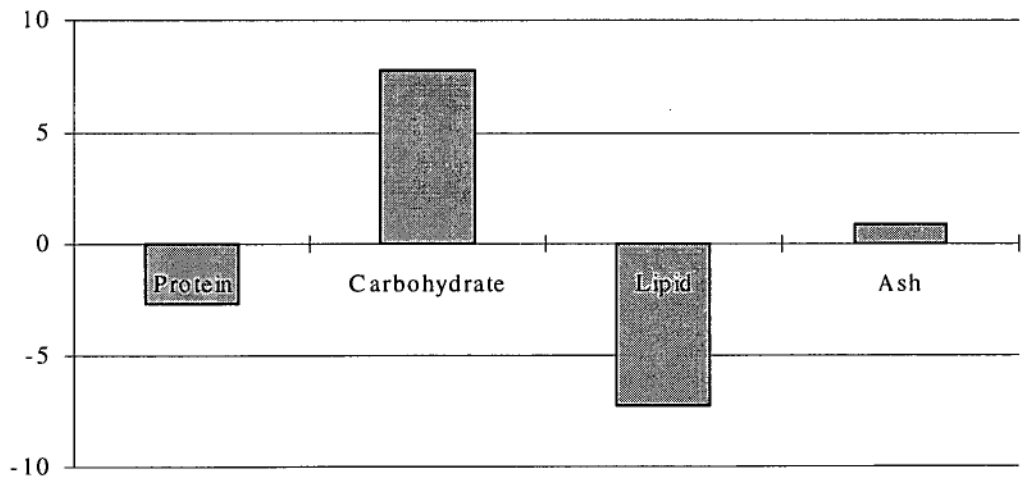
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Thalassiosira oceanica (CS-427)

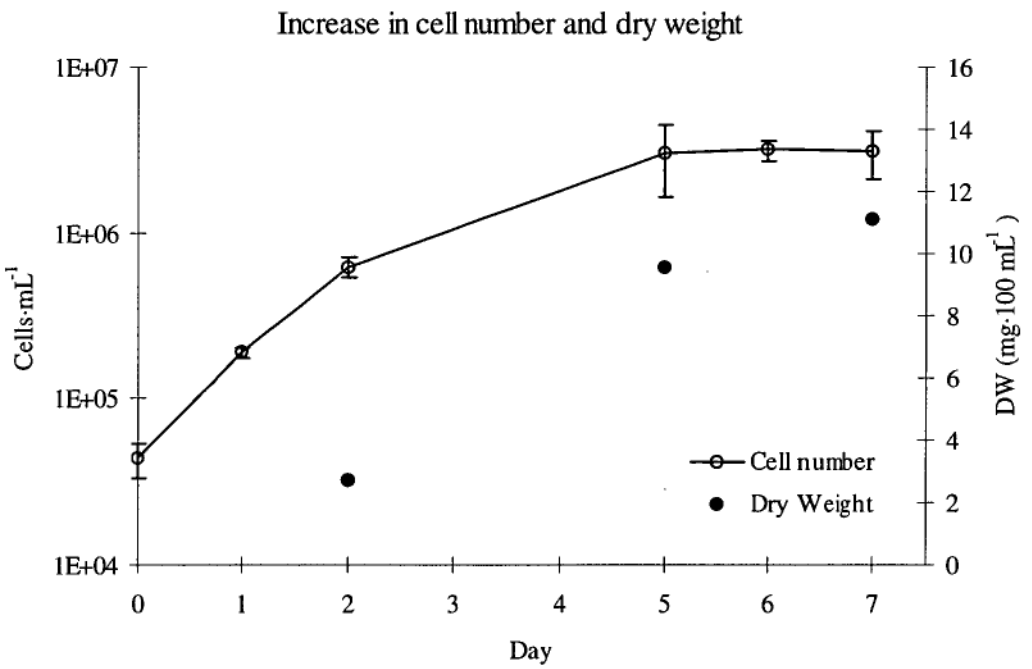
Growth data from triplicate cultures of *Thalassiosira oceanica* (CS-427). Exponential growth samples were taken on day 2, stationary phase samples on day 7.

Growth Statistics

Growth rate (μ)	**1.33**
Growth rate	**1.92** divisions·d^{-1}
Generation time	**0.52** days
Carrying capacity	**$3.11x10^6$** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·$cell^{-1}$)
0	$4.33x10^4 \pm 9.77x10^3$			
1	$1.90x10^5 \pm 1.42x10^4$			
2	$6.20x10^5 \pm 8.05x10^4$	2.72 ± 0.36	1.50 ± 0.25	44
5	$3.05x10^6 \pm 1.42x10^6$	9.57 ± 2.52	6.19 ± 0.46	31
6	$3.15x10^6 \pm 4.46x10^5$			
7	$3.12x10^6 \pm 1.04x10^6$	11.10 ± 3.20	8.33 ± 1.63	36



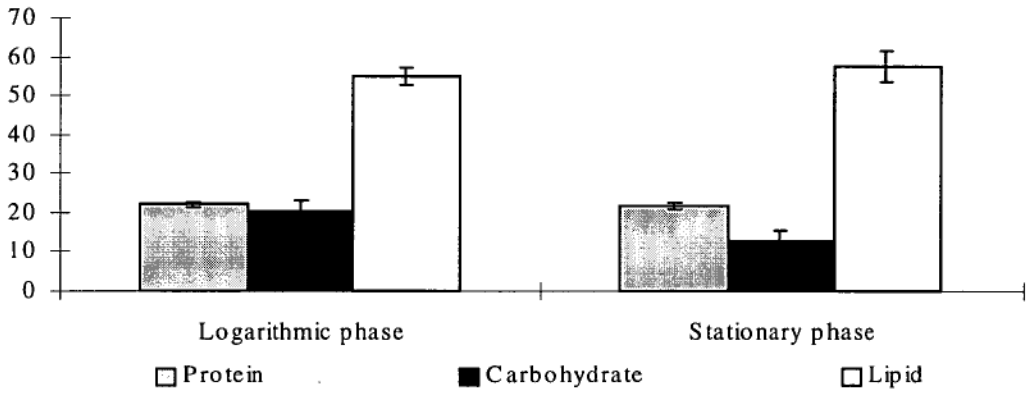
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	14.01 ± 1.07	12.90 ± 2.45	42.31 ± 2.59	36.25 ± 2.90	**105**
Stationary phase	17.22 ± 0.19	10.26 ± 2.07	45.96 ± 5.21	20.47 ± 3.32	**94**

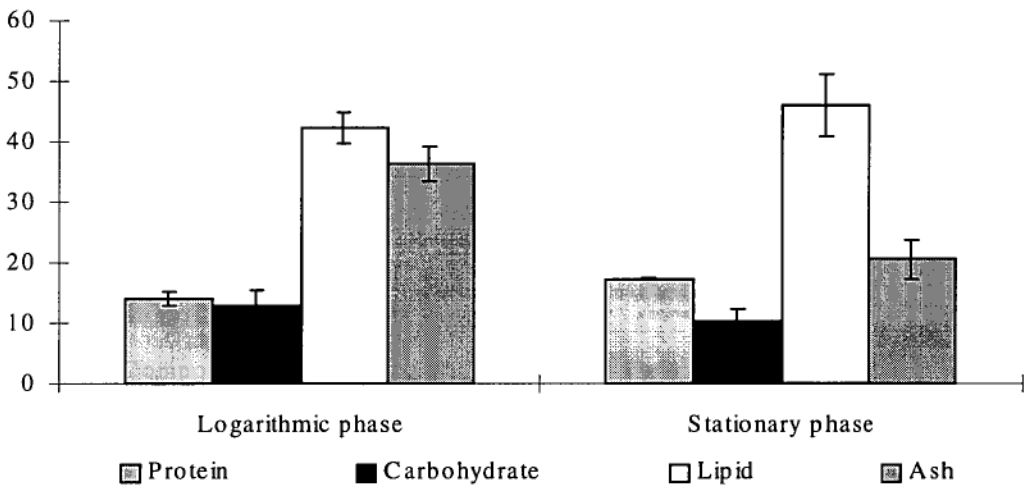
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	21.95 ± 0.77	20.16 ± 2.98	54.91 ± 2.39	**97**
Stationary phase	21.67 ± 0.72	12.93 ± 2.74	57.68 ± 4.06	**92**

Thalassiosira oceanica (CS-427)

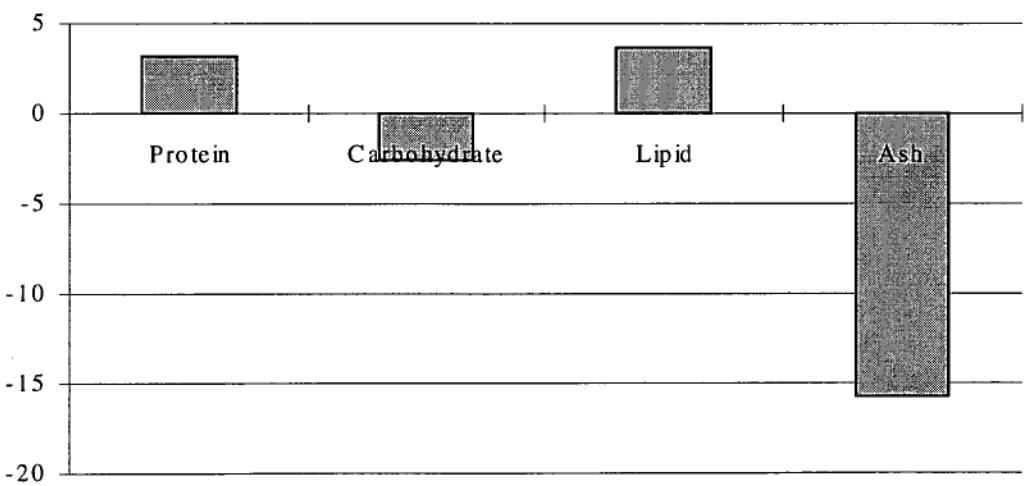
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Isolate CS-436 (*Chlorella* like)

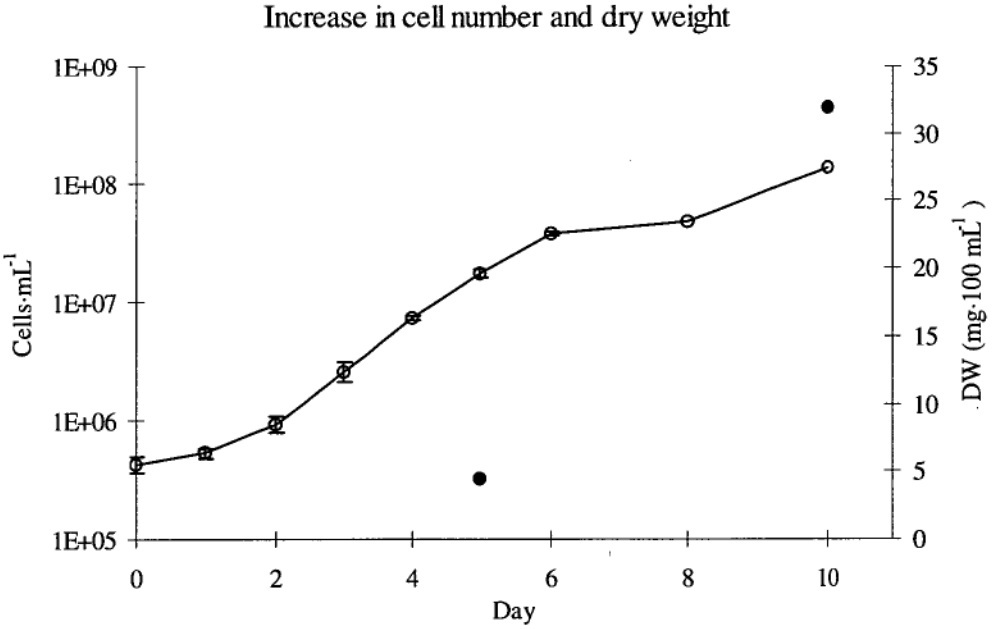
Growth data from triplicate cultures of isolate CS-436 (*Chlorella* like). Exponential growth samples were taken on day 5, stationary phase samples on day 10.

Growth Statistics

Growth rate (μ)	**0.93**
Growth rate	**1.34** divisions·d^{-1}
Generation time	**0.75** days
Carrying capacity	**$1.38x10^8$** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·$cell^{-1}$)
0	$4.23x10^5 \pm 6.66x10^4$			
1	$5.30x10^5 \pm 5.57x10^4$			
2	$9.40x10^5 \pm 1.45x10^5$			
3	$2.58x10^6 \pm 4.85x10^5$			
4	$7.31x10^6 \pm 1.64x10^5$			
5	$1.72x10^7 \pm 1.48x10^6$	4.41 ± 0.51	3.95 ± 0.51	3
6	$3.83x10^7 \pm 1.39x10^6$			
8	$4.85x10^7 \pm 3.50x10^6$			
10	$1.38x10^8 \pm 1.04x10^7$	31.88 ± 1.82	31.23 ± 1.77	2



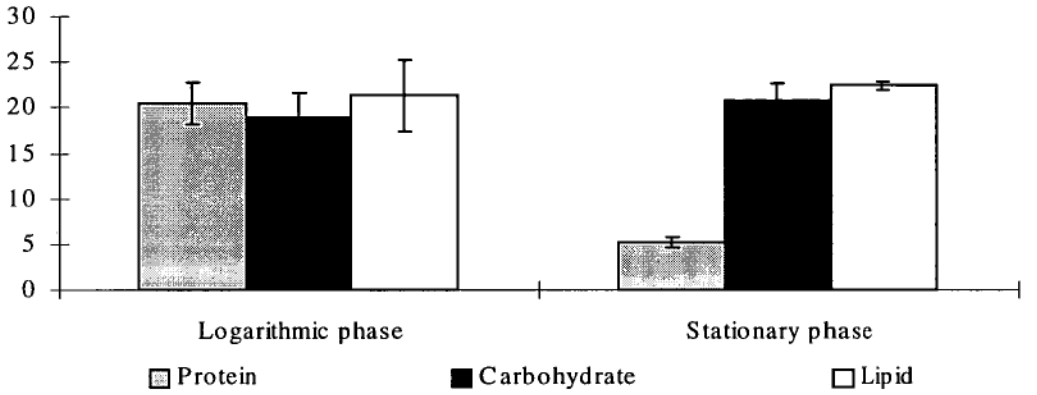
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	18.31 ± 1.83	16.98 ± 2.56	19.09 ± 3.29	10.51 ± 1.32	**65**
Stationary phase	5.03 ± 0.50	20.45 ± 1.93	22.04 ± 0.53	2.04 ± 0.99	**50**

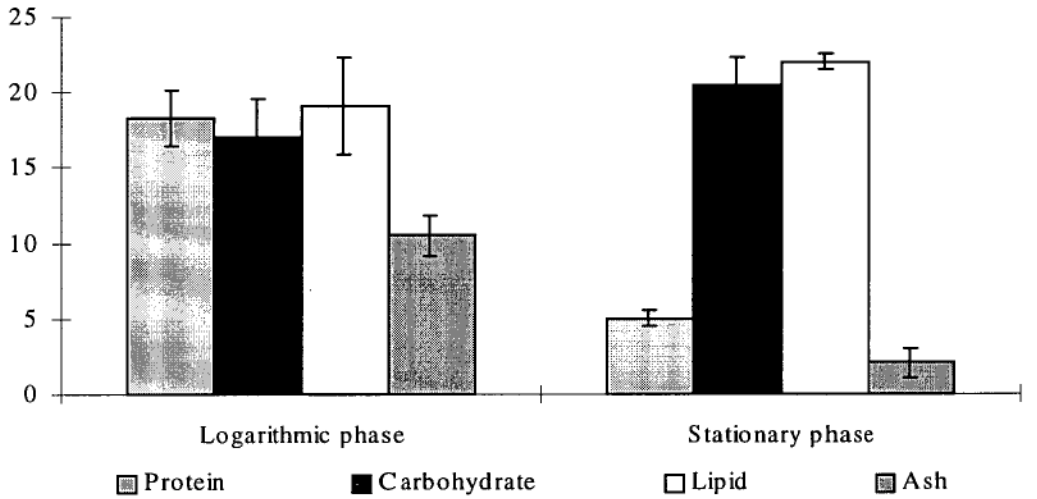
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	20.48 ± 2.28	18.95 ± 2.63	21.36 ± 3.94	**61**
Stationary phase	5.14 ± 0.56	20.86 ± 1.79	22.50 ± 0.53	**49**

Isolate CS-436 (*Chlorella* like)

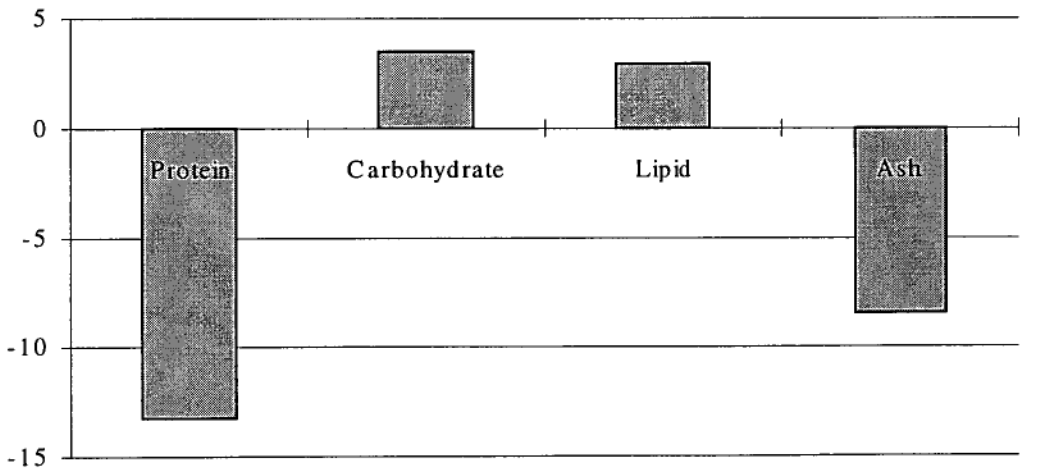
Composition (% AFDW) of logarithmic and stationary phase cultures



Composition (% DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Isolate CS-437 (*Stichococcus* like)

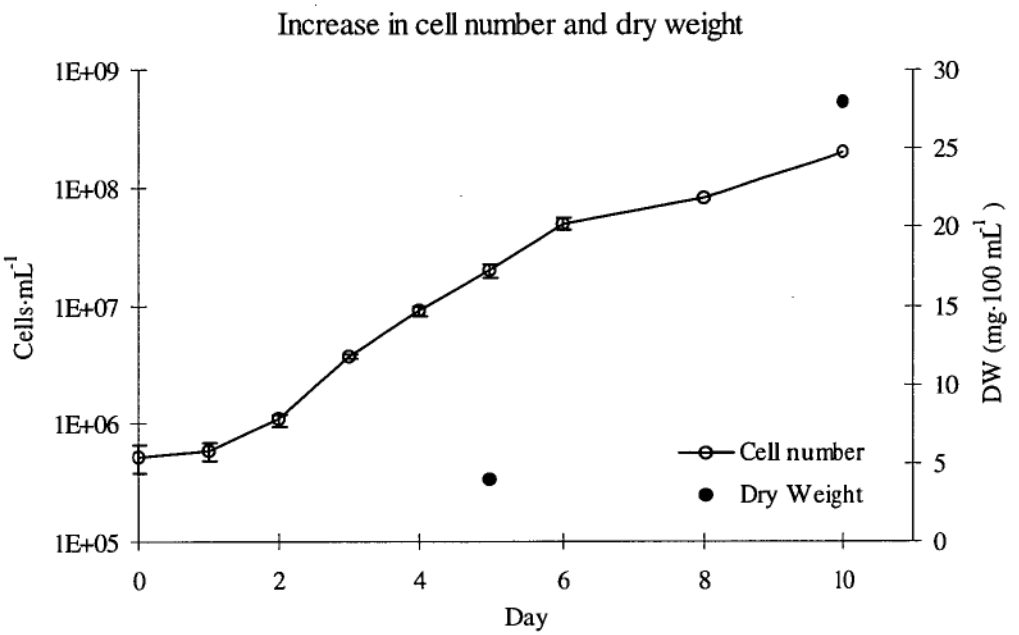
Growth data from triplicate cultures of isolate CS-437 (*Stichococcus* like). Exponential growth samples were taken on day 5, stationary phase samples on day 10.

Growth Statistics

Growth rate (μ)	**0.96**
Growth rate	**1.38** divisions·d^{-1}
Generation time	**0.73** days
Carrying capacity	**2.02x10^{8}** cells·mL^{-1}

Growth Data

Day	Cell concentration (cells·mL^{-1} ± S.D.)	DW (mg·100 mL^{-1} ± S.D.)	AFDW	Cell mass (pg·cell^{-1})
0	5.23x10^{5} ± 1.43x10^{5}			
1	5.87x10^{5} ± 1.00x10^{5}			
2	1.07x10^{6} ± 1.29x10^{5}			
3	3.63x10^{6} ± 1.61x10^{5}			
4	8.96x10^{6} ± 1.01x10^{6}			
5	1.98x10^{7} ± 2.88x10^{6}	3.92 ± 0.51	3.14 ± 0.41	2
6	4.90x10^{7} ± 5.45x10^{6}			
8	8.07x10^{7} ± 5.77x10^{5}			
10	2.00x10^{8} ± 1.17x10^{7}	27.94 ± 2.91	26.05 ± 1.29	1



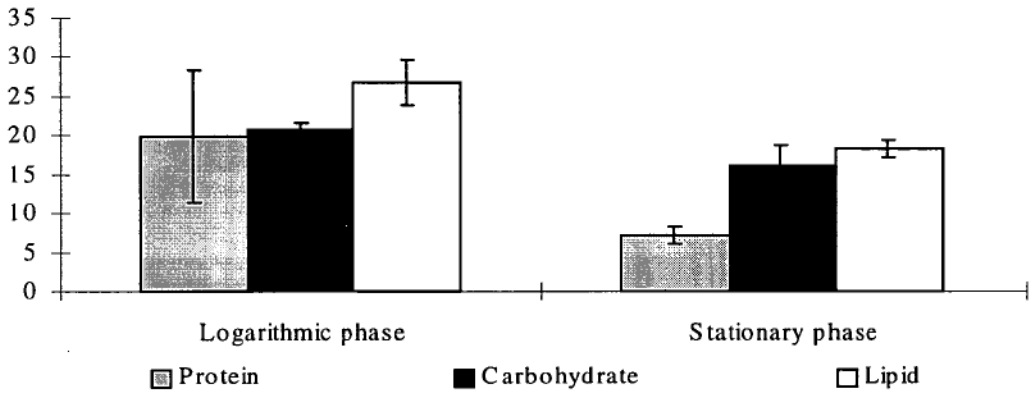
Percentage Composition

Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	Ash ± S.D.	**Total**
Logarithmic phase	15.84 ±6.50	16.65 ±0.91	21.45 ±2.49	19.93 ±1.06	**74**
Stationary phase	6.70 ±1.30	14.90 ±1.88	17.06 ±0.39	6.44 ±4.89	**45**

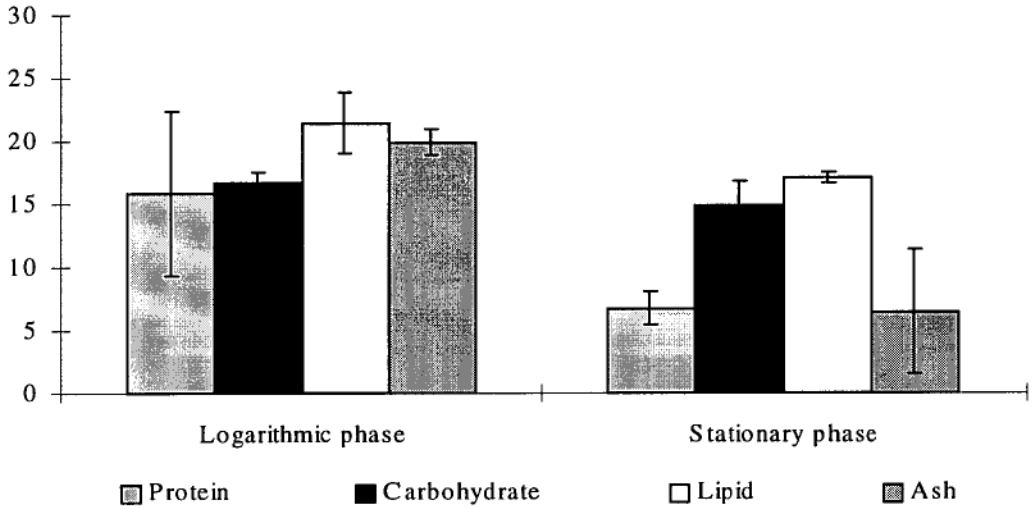
Ash Free Dry Weight	Protein ± S.D.	Carbohydrate ± S.D.	Lipid ± S.D.	**Total**
Logarithmic phase	19.85 ±8.41	20.79 ±0.92	26.77 ±2.85	**67**
Stationary phase	7.13 ±1.08	16.01 ±2.74	18.27 ±1.13	**41**

Isolate CS-437 (*Stichococcus* like)

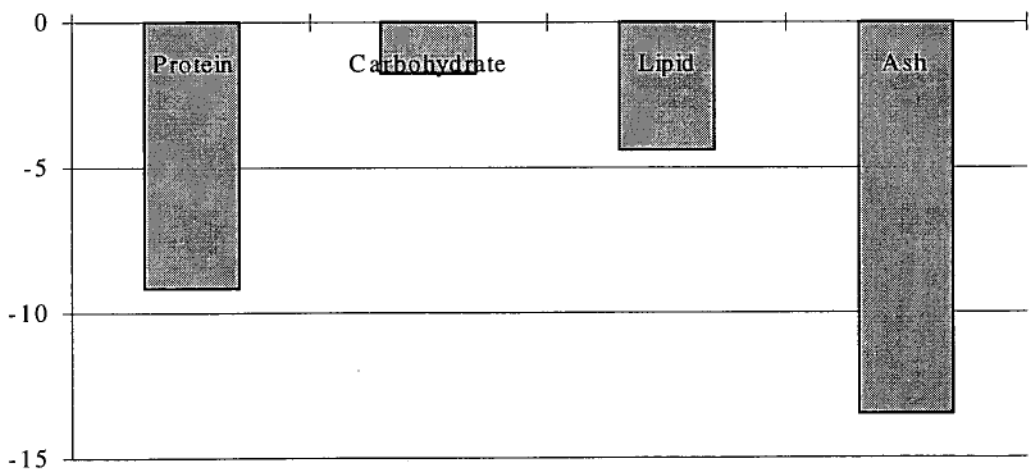
Composition (AFDW) of logarithmic and stationary phase cultures



Composition (DW) of logarithmic and stationary phase cultures



Change in the percentage composition from logarithmic to stationary phase cultures



Appendix Two

Fatty Acid Composition of Selected Australian Isolates

Fatty acid composition of extracted lipid from replicate cultures of *Thalassiosira oceanica* (CS-427).

	Percent of total fatty acids				Fatty acids: mg_100g^{-1} DW			
	Logarithmic		Stationary		Logarithmic		Stationary	
	Day 2		Day 7		Day 2		Day 7	
Saturated Fatty Acids								
14:0	3.35	3.14	4.60	4.69	71.5	79.4	1186.7	1195.6
15:0	0.67	0.70	0.10	0.61	14.2	17.8	25.9	154.9
16:0	19.41	18.02	29.75	30.42	414.6	455.6	7671.8	7757.8
18:0	1.22	1.21	0.44	0.47	26.1	30.7	112.9	120.5
other	0.62	0.31	0.08	0.07	13.2	7.9	20.0	16.7
total saturated	25.27	23.39	34.96	36.25	539.7	591.3	9017.2	9245.6
Branched-chain Fatty acids	0.88	0.38	0.28	0.20	18.7	9.7	71.4	52.0
Monoenoic Fatty Acids								
16:1(n-9)	0.22	0.58	0.05	0.02	4.6	14.6	12.2	6.1
16:1(n-7) (& 16:2(n-4) if present)	23.74	23.56	44.47	45.02	507.1	595.8	11469.8	11483.1
16:1(n-5)	0.21	0.20	0.23	0.24	4.4	5.0	59.4	60.3
16:1(n-13)t	0.40	0.36	0.25	0.23	8.6	9.1	64.8	59.6
18:1(n-9)	0.63	1.32	0.36	0.33	13.4	33.2	92.7	84.4
18:1(n-7)	0.24	0.47	0.29	0.27	5.2	11.9	73.8	68.5
other	0.15	0.31	0.06	0.07	3.2	7.8	15.8	16.9
total monoenoic	25.59	26.79	45.71	46.18	546.5	677.4	11788.4	11778.9
Polyenoic Fatty Acids								
16:4(n-1)	0.37	0.39	0.07	0.06	7.9	9.9	19.1	16.3
16:2(n-7) & 16:3(n-4)	11.43	13.17	4.30	3.88	244.1	332.9	1108.2	990.2
18:2(n-6)	0.75	0.71	0.52	0.45	16.1	18.0	134.0	114.2
18:3(n-3)	0.14	0.14	0.23	0.23	3.1	3.6	58.3	58.9
18:3(n-6)	0.14	0.00	0.00	0.00	3.0	0.0	0.0	0.0
18:4(n-3)	2.91	3.29	1.67	1.62	62.1	83.1	429.9	414.0
20:2(n-6)	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
20:3(n-6)	0.00	0.03	0.10	0.05	0.0	0.6	25.8	14.0
20:4(n-6)	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
20:4(n-3)	0.13	0.13	0.12	0.10	2.8	3.3	31.7	26.4
20:5(n-3)*	27.30	26.96	10.19	9.15	583.0	681.6	2628.0	2333.3
22:4(n-6)	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
22:5(n-3)	0.00	0.01	0.00	0.00	0.0	0.3	0.0	0.0
22:5(n-6)	0.36	0.33	0.36	0.38	7.8	8.3	93.9	96.1
22:6(n-3)#	3.88	3.65	1.19	1.19	82.9	92.2	306.0	304.4
other	0.02	0.08	0.03	0.00	0.5	1.9	7.7	0.0
total polyenoic	47.43	48.88	18.78	17.12	1013.2	1235.9	4842.6	4367.7
other un-id:								
?5 - prob i18:0	0.83	0.56	0.27	0.24	17.8	14.0	70.8	61.1
total un-id	0.83	0.56	0.27	0.24	17.8	14.0	70.8	61.1
total fatty acids	100	100	100	100	2135.9	2528.4	25790.5	25505.2

* includes 20:4(n-6) if "0" listed in table for this fatty acid.

\# includes 22:5(n-6) if "0" listed in table for this fatty acid.

other: fatty acid less than 0.1% in most samples

Fatty acid composition of extracted lipid from replicate cultures of *Entomoneis* cf. *punctulata* (CS-426).

	Percent of total fatty acids				Fatty acids: mg_100g -1 DW			
	Logarithmic		Stationary		Logarithmic		Stationary	
	Day 2		Day 7		Day 2		Day 7	
Saturated Fatty Acids								
14:0	10.62	11.75	12.59	12.39	293.7	375.8	1329.4	1260.6
15:0	0.60	0.52	0.41	0.37	16.5	16.6	42.8	37.3
16:0	16.44	15.69	18.59	15.25	454.6	501.6	1963.5	1551.8
18:0	1.73	1.45	0.83	0.29	47.8	46.2	87.4	29.8
other	0.54	0.44	0.17	0.18	14.8	14.1	18.0	18.3
total saturated	29.93	29.85	32.58	28.48	827.3	954.3	3441.1	2897.7
Branched-chain Fatty acids	0.39	0.46	0.21	0.60	10.7	14.7	22.3	61.2
Monoenoic Fatty Acids								
16:1(n-9)	1.18	0.66	0.34	0.49	32.7	21.1	36.0	50.0
16:1(n-7) (& 16:2(n-4) if present)	18.12	17.84	23.13	22.65	500.9	570.3	2443.2	2304.5
16:1(n-5)	0.36	0.38	0.21	0.24	10	12.1	21.8	24.5
16:1(n-13)t	0.43	0.40	0.35	0.30	12.0	12.7	37.3	30.8
18:1(n-9)	1.70	1.76	1.40	0.65	47.0	56.2	147.6	66.0
18:1(n-7)	0.33	0.31	0.15	0.16	9.1	9.8	16.3	15.8
other	0.30	0.27	0.19	0.20	8.4	8.6	20.6	20.3
total monoenoic	22.43	21.61	25.78	24.69	620.0	690.8	2722.8	2511.9
Polyenoic Fatty Acids								
16:4(n-1)	3.73	4.01	1.32	1.58	103.2	128.2	139.2	160.5
16:2(n-7) & 16:3(n-4)	7.44	7.23	7.14	8.54	205.6	231.1	754.0	868.8
18:2(n-6)	1.04	1.06	1.01	0.74	28.9	33.9	107.0	75.2
18:3(n-3)	0.25	0.23	0.31	0.24	6.9	7.3	32.5	24.0
18:3(n-6)	2.02	1.80	1.55	1.27	55.9	57.6	163.9	128.8
18:4(n-3)	6.29	6.48	5.12	4.78	173.8	207.3	540.6	486.6
20:2(n-6)	0.00	0.00	0.00	0.40	0.0	0.0	0.0	40.9
20:3(n-6)	0.02	0.02	0.00	0.00	0.7	0.7	0.0	0.0
20:4(n-6)	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
20:4(n-3)	0.44	0.45	0.61	0.57	12.2	14.3	64.3	57.7
20:5(n-3)*	24.86	25.52	23.15	26.60	687.3	815.8	2445.2	2706.6
22:4(n-6)	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
22:5(n-3)	0.99	1.18	1.18	1.39	27.3	37.6	124.8	141.7
22:5(n-6)	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
22:6(n-3)#	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
other	0.03	0.03	0.00	0.04	0.9	0.8	0.0	3.6
total polyenoic	47.11	48.00	41.39	46.14	1302.5	1534.7	4371.5	4694.5
other un-id:								
?5 - prob i18:0	0.14	0.09	0.04	0.08	4.0	2.7	4.6	8.5
total un-id	0.14	0.09	0.04	0.08	4.0	2.7	4.6	8.5
total fatty acids	100	100	100	100	2764.6	3197.2	10562.3	10173.8

* includes 20:4(n-6) if "0" listed in table for this fatty acid.

\# includes 22:5(n-6) if "0" listed in table for this fatty acid.

other: fatty acid less than 0.1% in most samples

Fatty acid composition of extracted lipid from replicate cultures of *Extubocellulus spinifera* (CS-428).

	Percent of total fatty acids				Fatty acids: mg_100g -1 DW			
	Logarithmic		Stationary		Logarithmic		Stationary	
	Day 3		Day 7		Day 3		Day 7	
Saturated Fatty Acids								
14:0	8.63	6.76	15.23	16.15	301.5	252.2	1629.7	2315.5
15:0	0.44	0.43	0.69	0.57	15.3	16.0	73.8	81.5
16:0	10.23	12.75	16.66	17.43	357.4	475.8	1782.2	2499.3
18:0	0.65	0.99	0.16	0.25	22.8	37.0	16.8	36.4
other	0.47	0.39	0.08	0.17	16.3	14.7	9.1	24.3
total saturated	20.41	21.32	32.82	34.56	713.3	795.6	3511.5	4957.1
Branched-chain Fatty acids	1.23	0.97	0.35	0.27	43.0	36.3	37.1	38.7
Monoenoic Fatty Acids								
16:1(n-9)	0.49	0.11	0.04	0.04	17.2	4.1	4.1	6.1
16:1(n-7) (& 16:2(n-4) if present)	28.90	27.44	40.58	40.24	1009.7	1024.0	4342.1	5770.5
16:1(n-5)	0.12	0.10	0.39	0.39	4.1	3.8	41.6	56.1
16:1(n-13)t	0.07	0.05	0.06	0.02	2.4	1.7	6.0	2.7
18:1(n-9)	0.52	1.44	2.19	1.85	18.3	53.7	234.0	265.4
18:1(n-7)	0.26	0.50	0.15	0.20	9.1	18.8	15.9	29.2
other	0.27	0.30	0.22	0.24	9.5	11.3	23.3	34.2
total monoenoic	30.63	29.94	43.62	42.98	1070.3	1117.2	4666.9	6164.2
Polyenoic Fatty Acids								
16:4(n-1)	0.49	0.25	0.12	0.09	17.2	9.4	13.3	12.7
16:2(n-7) & 16:3(n-4)	8.80	8.59	4.02	3.94	307.6	320.6	430.5	564.4
18:2(n-6)	0.69	0.46	0.73	0.55	24.2	17.2	77.7	78.9
18:3(n-3)	0.05	0.04	0.07	0.03	1.9	1.5	7.0	4.4
18:3(n-6)	0.47	0.33	0.22	0.19	16.4	12.2	23.8	27.0
18:4(n-3)	0.34	0.26	0.32	0.19	12.1	9.7	34.8	27.1
20:2(n-6)	0.44	0.02	0.14	0.15	15.2	0.6	15.0	21.5
20:3(n-6)	0.17	0.10	0.12	0.14	6.1	3.8	13.2	20.3
20:4(n-6)	10.71	8.99	0.00	0.00	374.2	335.5	0.0	0.0
20:4(n-3)	0.10	0.04	0.09	0.10	3.7	1.6	9.6	14.9
20:5(n-3)*	22.04	24.18	15.41	14.98	770.2	902.3	1649.3	2148.8
22:4(n-6)	0.09	1.03	0.00	0.00	3.1	38.6	0.0	0.0
22:5(n-3)	0.12	0.03	0.05	0.03	4.1	1.1	5.7	4.9
22:5(n-6)	0.28	0.24	0.44	0.35	9.7	9.0	46.8	50.0
22:6(n-3)#	2.58	2.49	1.38	1.27	90.2	93.1	147.4	182.5
other	0.02	0.00	0.02	0.00	0.6	0.0	2.0	0.0
total polyenoic	47.40	47.06	23.14	22.02	1656.3	1756.2	2476.1	3157.3
other un-id:								
?5 - prob i18:0	0.33	0.72	0.08	0.17	11.4	26.7	8.5	24.2
total un-id	0.33	0.72	0.08	0.17	11.4	26.7	8.5	24.2
total fatty acids	100	100	100	100	3494.3	3732.1	10700.2	14341.6

* includes 20:4(n-6) if "0" listed in table for this fatty acid.

\# includes 22:5(n-6) if "0" listed in table for this fatty acid.

other: fatty acid less than 0.1% in most samples

Fatty acid composition of extracted lipid from replicate cultures of *Attheya septentrionalis* (CS-425).

	Percent of total fatty acids				Fatty acids: mg_100g -1 DW			
	Logarithmic		Stationary		Logarithmic		Stationary	
	Day 3		Day 7		Day 3		Day 7	
Saturated Fatty Acids								
14:0	14.55	14.36	20.89	22.05	927.8	489.4	3926.9	4281.4
15:0	0.27	0.30	0.28	0.31	17.4	10.3	53.5	59.9
16:0	6.66	7.95	15.41	13.50	425.0	271.0	2896.9	2621.1
18:0	1.08	1.16	0.97	0.71	69.0	39.4	181.7	137.5
other	0.81	0.51	0.30	0.23	51.7	17.5	56.3	44.3
total saturated	23.38	24.28	37.86	36.79	1490.9	827.6	7115.4	7144.3
Branched-chain Fatty acids	0.14	0.15	0.33	0.34	8.8	5.2	61.5	65.4
Monoenoic Fatty Acids								
16:1(n-9)	0.30	0.69	0.05	0.08	19.4	23.6	9.5	15.1
16:1(n-7) (& 16:2(n-4) if present)	21.46	21.54	28.97	29.39	1368.5	734.1	5445.0	5707.7
16:1(n-5)	0.21	0.20	0.18	0.23	13.6	6.9	33.7	43.8
16:1(n-13)t	0.16	0.21	0.11	0.12	10.2	7.2	20.0	22.6
18:1(n-9)	0.41	0.40	0.58	0.41	26.2	13.6	108.5	79.4
18:1(n-7)	0.88	0.80	0.26	0.23	56.1	27.4	49.6	44.7
other	0.02	0.13	0.10	0.25	1.4	4.4	18.5	48.4
total monoenoic	23.45	23.98	30.25	30.70	1495.4	817.3	5684.9	5961.6
Polyenoic Fatty Acids								
16:4(n-1)	2.29	1.66	0.54	0.50	146.3	56.6	102.0	96.7
16:2(n-7) & 16:3(n-4)	14.47	14.08	3.47	3.90	922.7	479.9	651.5	757.9
18:2(n-6)	1.06	1.10	0.82	0.72	67.9	37.6	153.6	140.6
18:3(n-3)	0.06	0.06	0.13	0.14	3.8	2.1	23.8	27.5
18:3(n-6)	0.56	0.75	0.38	0.35	35.8	25.6	72.2	68.8
18:4(n-3)	0.58	0.65	0.81	0.81	37.2	22.1	152.3	158.1
20:2(n-6)	0.03	0.00	0.24	0.30	2.0	0.0	45.7	57.6
20:3(n-6)	0.16	0.09	0.41	0.40	10.5	3.1	77.2	78.4
20:4(n-6)	0.00	0.00	0.00	0.00	0.0	0.0	0.0	0.0
20:4(n-3)	0.18	0.09	0.40	0.49	11.4	2.9	75.5	94.7
20:5(n-3)*	27.10	26.83	22.11	22.18	1727.7	914.4	4155.4	4307.1
22:4(n-6)	0.05	0.05	0.00	0.00	3.3	1.6	0.0	0.0
22:5(n-3)	0.08	0.05	0.09	0.06	5.2	1.7	16.0	12.0
22:5(n-6)	0.83	1.64	0.22	0.34	52.9	56.0	40.5	65.3
22:6(n-3)#	4.96	4.06	1.83	1.87	316.6	138.3	344.7	363.1
other	0.00	0.01	0.00	0.00	0.0	0.4	0.0	0.0
total polyenoic	52.43	51.12	31.45	32.07	3343.1	1742.3	5910.5	6227.7
other un-id:								
?5 - prob i18:0	0.60	0.46	0.12	0.10	38.2	15.7	23.1	20.4
total un-id	0.60	0.46	0.12	0.10	38.2	15.7	23.1	20.4
total fatty acids	100	100	100	100	6376.3	3407.9	18795.4	19419.4

* includes 20:4(n-6) if "0" listed in table for this fatty acid.

\# includes 22:5(n-6) if "0" listed in table for this fatty acid.

other: fatty acid less than 0.1% in most samples